Potentiometric and Spectrophotometric Investigation of Copper Diclofenac as an anti-inflammatory drug for the treatment of Rheumatoid Arthritis.

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Before everything else, getting ready is the secret to success. Keep your face to the sunshine and you will not see the shadows, so don't be afraid to open up your eyes so you can see tomorrow

....UNKNOWN

Declaration

This piece of work is entirely my contribution and no ones results shown. I carried all of the results shown in this research all by my own at the University of Cape Town, therefore these are all originals no previous publications taken.

Compiled and edited by:

Nelisa P.V.Nama (Miss)

Signed by candidate

Dedication

I dedicate this entire dissertation to you my mother you are everything to me. This is nothing compared to the care and love you have given me but accept it as a small present saying thank you as it comes deep down my heart. Wait for what you sow, ndiyabulela Dlamini nangamso!!

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May the Almighty God, my Saviour Jesus Christ help you all keep the good work you have shown on me even to other people, with those words I thank you all.

Abstract

Copper and Diclofenac (DCL) separately, are used to alleviate inflammation associated with RA. It has been postulated that their combination would have a synergistic effect i.e., their combination would be more effective than when administered independently. Cu(II)-DCL system is studied potentiometrically in order to evaluate this hypothesis. Because of solubility limitations the system was studied in 50% MeOH/H_2O and in water with β-cyclodextrin (β-CD) as a carrier molecule. Protonation constants of 4.82 and 4.29 were obtained in a 50% MeOH/H_2O mixture and β-CD solution respectively. Based on pKa values, and the pH at which precipitation occurred, the solubility products of 5.5 $x10^{-8}M^2$ and 6.9 $x10^{-8}M^2$ were calculated for the protonated DCL in the two media respectively. The improvement in solubility products is due to the difference in protonation constants for the two media used.

Because of solubility problems, it was not possible to investigate the copper complex of the ligand using the β-CD environment. The only log stability constants obtained were 3.48 and –3.02 for the 1 1 0 and 1 1 -1 species respectively in 50% MeOH/H_2O. Computer simulation of intestinal absorption indicates that the CuDCL complex would exist at pH > 5 which improve the bioavailability of both the copper and DCL. However blood plasma simulation studies indicated that the ligand is devoid of any Cu(II) mobilising ability. DCL is then unable to increase the low molecular weight Cu(II) fraction in blood plasma.

Abbreviations

β-CD	Beta cyclodextrin
CD	Cyclodextrin
COX	Cyclooxygenase
DCL	Diclofenac
DMARD	Disease modifying antirheumatic drug
ECCLES	Evaluation of constituent concentrations in large equilibrium systems
Emf	Electromagnetic force
ESTA	Equilibrium simulation for titration analysis
GEP	Glass Electrode Potentiometry
Gly	Glycine
MeOH\H_2O	Methanol\water
NMR	Nuclear magnetic resonance
NSAID	Non steroidal anti-inflammatory drug
OBJE	ESTA task for optimising titration parameters with respect to *emf* titration points
PG	Prostaglandin
p.m.i	Plasma mobilising index
QBAR	ESTA task for calculating the deprotonation function
RA	Rheumatoid Arthritis
SOD	Superoxide dismutase
SPEC	ESTA task for calculating the species distribution of the component chemical species
TB	Tuberculosis
ZBAR	ESTA task for calculating formation constants

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CHAPTER ONE

INTRODUCTION

1. Introduction

Inflammation is an important response to tissue injury due to any cause. The importance of this multifaceted process is best appreciated as the beginning of the tissue repair process which is required to re-establish normal function.[1] Persistent inflammation in the absence of tissue repair and the resultant lack of normal function is recognised as chronic inflammation. An "anti-inflammatory" agent that inhibits some facet of the inflammatory process and, as a result, the repair process would not be expected to re-establish normal function. Alternatively, an anti-inflammatory agent which facilitates the repair process would be expected to re-establish normal function.[1] Similarly, inflammation can affect the lining of the joints so that they too become hot, swollen and painful. The joint then becomes difficult to move and does not bend as far as it should.[2] Eventually the joint may be considerably damaged and internal scarring may stop it moving freely. The most common cause of inflammation of this type occurring in several joints in one person is Rheumatoid Arthritis (RA).

1.1 Rheumatoid Arthritis (RA)

Rheumatoid Arthritis (RA), is a debilitating disease, afflicting some 5% of the Western World's population and there is no cure yet.[1] RA is the most severe of the arthritis disorders. The word "Arthritis" simple means inflammation of the joints.[2]

In the normal synovial joint, articular cartilage covers the bone ends and both are enclosed by a synovial membrane. Articular cartilage is the dense tissue containing collagen fibres and cells (chondrocytes) embedded in a matrix of proteoglycan. The normal synovial lining of the joint exists as thin fibrous tissue which is made up of lining cells (synoviocytes) overlying fatty and fibrous material.

The synovial lining synthesizes the polymer hyaluronic acid and secretes it into the synovial fluid, where it is largely responsible for the viscosity of the fluid. The synovial lining also acts as a barrier to the free movement of proteins from plasma into the synovial fluid.[4]

RA is a common condition. It affects something like one in every fifty of the adult population, but fortunately most of these people are only mildly affected.[3] Indeed some have such a mild attack that they do not feel ill enough to consult their doctors.

They noticed temporary pain and swelling in their joints, perhaps in their fingers, but after a few weeks the symptoms disappear and there is no further trouble.[3] For others, however, the condition does not settle down and it is they who seek medical help. A minority of this group who are more seriously affected go on to develop severe deformities and become crippled.[3]

RA is much more common in women than in men. For every man affected there are two or three women with this disease. The reason for this difference is not clear.[3] RA usually begins between the age of thirty and sixty but there is evidence that it can start at any age. It has been recorded that the disease involved an infant of only nine months and at the other extreme of life span, the condition can develop for the first time in elderly people, even in those over the age of ninety.[3]

1.1.1 What is the cause?

It has been said "...everything is known about RA except its cause, natural history and treatment. It is one of the perversities of the human condition that the rarer the disease, the more clearly it seems to be understood".[5]

The body inadvertently alters its defence mechanisms so as to attack its own linings i.e. the synovium. The explanation comes from the observation that the joint linings show changes which are similar to those seen in any part of the body in which an "antibody" reaction is involved, as in resisting a virus.[3]

1.1.2 What happens in RA

The onset of rheumatoid arthritis is usually slow.[6] The first noticeable changes are swelling and inflammation of the lining of the joint-synovium. This becomes abnormally thick and swollen due to the persistent inflammation, and eventually it grows and spreads over the surface of the joint.[6] Its permeability increases, so that more plasma contents enter the joint. Iron deposition is seen within the synovial cells; much of this iron occurs within ferritin and haemosiderin.[6]

In many patients with rheumatoid arthritis, the disease leads to the destruction of the articular cartilage, bone erosion, and impairment of joint function.[6] Here it can, if it goes on long enough, damage the underlying cartilage, which is the natural load-bearing surface of the joint. Eventually it "eats" its way through, causing irreversible damage to the normal smooth slippery joint surface.[3]

The ligaments which provide stability to the joint are also weakened so that not only is the efficiency of the joint greatly diminished but it also becomes unstable.[3] Damage and pain can ensue because the joint is moved in directions or degrees for which it was not originally designed. Inflammation can also occur in tissues under the skin particularly in the forearms just below the elbow joint.[3]

This is the result of leaning forwards on the forearms when resting at a table. Small lumps called R nodules develop which can grow and become tender [3] This means that there may be changes elsewhere.[5] The first symptoms of arthritis are often just generalised aches and pains. Stiffness of the hands and the other joint is a common feature. This is noticeable first thing in the morning and it may take several hours to disappear. At this stage sufferers complain more of this than of pain.[6]

Later with the further development of arthritis, the joints became swollen and painful. When pressed upon they feel tender and an attempt to use them generates pain. Because of this, the sufferer tries to avoid using the affected joints with the danger that movement becomes more and more restricted.

There are certain joints, which are especially prone to be affected by RA. These are the finger joints, the wrist, knees and those at the bases of the toes.[6] The disease can occur in joints such as the ankles, elbows, shoulders and hips, but they are affected less frequently. [6]

1.2 Treatment of RA

A massive research program has been launched in the last few years in an attempt to determine the cause of RA.[3] Indeed the pace of medical research is such that we reasonably hope to find the cure for RA one day.

But even if we cannot talk of a cure yet, there are several methods of treating this disease which are important as means of providing relief from pain, preventing unnecessary deformities and enabling the sufferer to be as independent as possible.[3] These treatments are divided into rest, occupational therapy aids, splints and plasters, physiotherapy, drugs and surgery.[4] Two of these are discussed below.

(i) The importance of rest:

There is no doubt that periods of rest are of the greatest value to patients with RA. When the disease is really severe then complete bed rest is essential. People are often admitted to hospital purely for this reason alone.[3]

(ii) Medical control of RA

Since RA cannot be cured, it can only be alleviated also by drug therapy. Drugs are foreign compounds to the body and therefore they have to be administered very carefully in order to avoid unnecessary side effects.

There are drugs that are aimed at inhibiting the pain and suppress inflammation and are widely used for arthritis. These are non-steroidal anti-inflammatory drugs (NSAIDs), disease-modifying antirhemautic drugs (DMARDs) and glucocorticoids.

(a) NSAIDs.

The actual mechanisms of drug action are complicated, but some explanation can make understanding them easier.[6] NSAIDs are often essential agents in the management of arthritis and have a major role in the management of pain, but they also may be commonly employed for conditions that are less serious. NSAIDs work quickly when they are given and the effects wear off quickly when they are withdrawn.[7]

Aspirin[3] (acetylsalicylic acid) is a familiar drug for relieving pain and as such it is the most useful drug for treating RA. If taken in the full dosage it will actually suppress the inflammation. Also, many clinicians believe that higher doses of these NSAIDs are more effective than lower doses for the treatment of RA but are associated with higher rates of adverse events.[17] There are also other (NSAIDs)[8] ibuprofen and acetaminophen. These are widely used for their ability to decrease inflammation and pain.[8]

Examples of NSAIDs are shown in Table 1 below and also the next class of drugs employed in the medical control of RA is DMARD shown in Table 2 below. These drugs produce remissions of RA.

Table1: Non steroidal anti-inflammatory drugs[8]

Anti-inflammatory, Analgesic, Antipyretic	*Anti-inflammatory, Analgesic*	*Anti-inflammatory*	*Analgesic*
Aspirin	*Diflunisal*	Choline-Mg-trisilicate	*Fenoprofen*
Salsate	*Azapropazone*	*Piroxicam*	*Isoxicam*
Phenylbutazone	*Tolmetin*	*Orgotein*	*Etodolac*
Indomethacin	*Fenbufen*		
Mefenamic acid	*Tiaprofenic*		
Diclofenac	Meclofenamate sodium		
Suprofen	*Flufenamic acid*		
	Tenoxicam		
	Ibuprofen		
	Naproxen		
	Ketoprofen		
	Flurbiprofen		

(b) DMARDs

These drugs are aimed not only in treating the symptoms but also at suppressing the disease at the source by diverting the course of the pathologic reactions.[9] The drug has to be administered over a long period of time because remission may occur at termination of therapy.

Table 2: Disease modifying antirheumatic drugs[8]

DRUG	*DOSAGE*	*ASSIMILATION*	*SIDE EFFECTS*
Sodiumauriothiomalate (Gold salt)	50 mg.1x week	Injected into muscles and excreted in urine and faeces.	*Skin rash, proteinuria and* blood dyscrasia
Penicillamine	125 – 250 mg per day	Orally administered, well absorbed in the stomach. Excreted mostly in and little in faeces.	*Low marrow* cell count and nausea
Azathioprine	*100mg/day in 3 doses.* 150mg/day if response is slow.	Orally administered and excreted in urine and faeces.	*Less toxic than* Other Immunosuppressive agents.
Chloroquine and Hydroxychloroquine	Adults: 200-250 mg /day ChlQ. 400-600 mg /dayHChlQ. Children: 3 mg/day ChlQ. 5 mg /dayHChlQ.	*Orally* administered, absorbed in the alimentary canal and excreted in the urine.	*Visual impairment* due to retinal damage

There are also synthetic corticosteroids that form part of RA drug regimen.[9] These are steroid and cortisone preparations.[3] When naturally occurring in the body they take part in many physiological chemical reactions. Example of such drugs, which is of relevance to the management of RA, is glucocorticoids.[9]

(c) Glucocorticoids

These have been synthesised and made into preparations for use in the treatment of tissue inflammation. They act to reduce heat, swelling and tenderness at the inflamed joint. Even though their mode of action is unclear, they have proved to be the best at combating inflammation. There is however, one overriding disadvantage in the use of corticosteroids for RA treatment. They are neither antiviral nor antibacterial.[10] They may seem to be very effective at the alleviation of the RA symptoms whilst they are actually obscuring the damage done by microbes in the body.

1.3 Prostaglandins in RA treatment.

In 1971 J. R. Vane[11] proposed the hypothesis that the anti-inflammatory effects of aspirin were due to its ability to inhibit prostaglandin synthesis. The discovery by Vane that both the therapeutic and the toxic effects of NSAIDs are produced by their action in preventing the synthesis of prostaglandins (PGs) by inhibition of COX enzymes was undoubtedly one of the landmark discoveries of the century for the pharmaceutical industry.[13]

These mechanisms do not explain, however, why different drugs administered at equi-active therapeutic doses exhibit side effects of different severity, and why there is limited correlation between inhibition of PG biosynthesis and anti-inflammatory activity. However, inhibition of cyclooxygenase (COX) activity is only one action of NSAIDs.[11]

1.4 The role of Cyclooxygenase (COX) in inflammation.

Recently, two different COX isoforms have been characterised: COX-1 and COX-2.[12] Inhibition of COX-1 and/ COX-2 leads to very different pharmacological effects. The COX-1 inhibition is predominantly responsible for anti-thrombotic effects and is expressed in all tissues while anti-inflammatory effects are mediated mainly by COX-2 and is predominant in the kidney, brain and ovaries.[12]

Both these COX isoenzymes are present in human rheumatoid synovial fluid, but only COX2 is regulated by interleukin-1.[13] Human rheumatoid joints contain many inflammatory cytokines which also up-regulate COX2 expression.[13] When a cell membrane is injured, arachidonic acid is released into the cell where it is oxidised (broken down) by COX, enzymes found in the cell membrane.[12]

The by-products of this reaction are called endoperoxides and are converted into PGs. In inflamed tissue, PGs produced by COX2 cause a variety of effects including fever, swelling, redness, accumulation of white blood cells, and stimulation of bone resorption.[14] Its mode of action is suggested to be the inhibition of COX-2, which is the cytikine inflammatory inducible enzyme, while its action on the physiologically responsive COX-1 is minimal.[12]

NSAIDs decrease injury by acting as anti-oxidants as well as blocking PG formation by inhibiting COX2 enzymes, which decrease fever, swelling, and pain.[15] These side effects are why other medications are prescribed in addition and is probably the inhibition of this enzyme activity by NSAIDs that produces the dramatic clinical response.[16]

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CHAPTER TWO

MOTIVATIONAL STUDY

2. Motivational Study

2.1 Background on the Study

2.1.1 Diclofenac in RA.

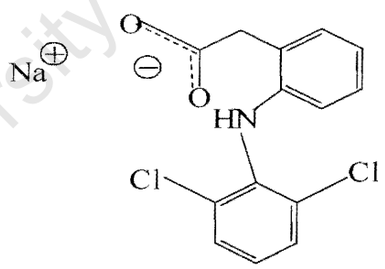
Diclofenac (DCL) is a potential NSAID, therapeutically used in inflammatory diseases of rheumatic and non-rheumatic origin.[1] It relieves pain and reduces swelling in the treatment of rheumatoid arthritic, (including arthritis in children), osteoarthritic and ankylosing spondylitic (inflammation of the joints between the spine and pelvis) patients.[2] Its action on the response of COX-1 is minimal. In vitro inhibition of COX by DCL does not translate to in vivo inhibition. Diclofenac is also found to decrease prostaglandins directly in synovial fluid in humans and in urine and renal medula in rats and pigs.[3]

Diclofenac has been found to interact in vivo with arachidonic acid cascade at the level of COX.[3] The anti-inflammatory activity of diclofenac and most of its other pharmacological effects are thought to be related to the inhibition of the conversion of arachidonic acid to prostaglandins, which are the mediators of the inflammatory process.[4] Like other NSAIDs, diclofenac is highly (>95%) protein bound.[5] DCL is also a potential reversible inhibitor of the secondary phase of induced platelet aggregation.[5]

Diclofenac (Figure 1 below), as the sodium salt, is a benzene acetic acid derivative. It is designated chemically as sodium 2-[(2,6-dichlorophenyl) amino] benzene acetic acid.[6] The structure of diclofenac consists of a phenyl acetic acid group, a secondary amino group, and a phenyl ring, both ortho positions are occupied by chlorine atoms causing a torsion angle of 70 and 69 respectively between the two aromatic rings. It is freely soluble in methanol, soluble in ethanol and sparingly soluble in water.[6]

Diclofenac, like other drugs of its class, is not free of side effects. The side effects of these drugs can cause discomfort and rarely, more serious side effects, such as gastrointestinal bleeding, liver toxicity, which may result in hospitalisation and even fatal outcomes.[5]

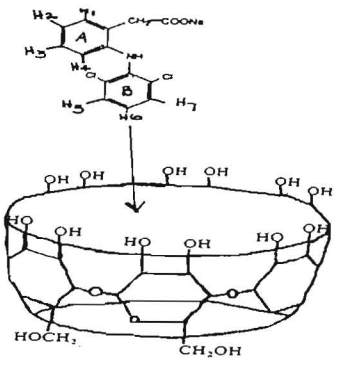
Figure 1a. The structure of sodium Diclofenac.



2.1.2 Role of Cyclodextrins in pharmaceutical industry.

In the last few years, pharmaceutical modification of drug molecules by inclusion has been extensively developed to improve their solubility, chemical stability, absorption and bioavailability.[7] Microencapsulation of drug molecules in cyclodextrins (CDs) has been extensively used in the pharmaceutical industry to produce more stable drug preparations with improved bioavailability.[8] Host-guest interactions in CD complexes include hydrogen bonding, hydrophobic binding and polar interactions, some or all of which may contribute to complex stabilization, depending on the chemical nature of the guest and that of the CD. CDs are cyclo amyloses containing α-CD and β-CD having the polar hydrophilic outside and a relatively non-polar lipophilic inside which lead naturally to two important results: - (i) using its lipophilic interior, a CD typically takes as a guest, a non-polar organic molecule or the non-polar end of an organic molecule. (ii) Its hydrophilic exterior, on the other hand, confers water solubility to the resulting complex (as shown by Figure 1b).

Figure 1b. Cyclodextrin inclusion of Diclofenac



2.1.3 β-CD

The inclusion complexation of drug molecules by β-CD has been extensively studied.[9] It has been found that β-CD greatly improves the pH dependent solubility of NSAIDs. These have been used in various ways such as to accelerate or retard organic reactions or as simple models of enzymatic reactions. β-CD has been applied to numerous reactions related to hydrolysis and decarboxylation.[9] In the present work, the guest-host complexation has been investigated by using potentiometry.

2.1.4 Copper Activity in RA.

The relationship of copper to RA is complex,[10] it has been linked to RA for centuries. Folk-lore treatment of arthritis invariably involves copper, either in a copper-rich diet such as shellfish, nuts and cider vinegar, or the ubiquitous copper bangle.[24] Walker et al.[25, 11] have measured the dermal absorption of copper from bracelets and found it to be significant. This has been shown by counting the activity that the copper(II) complex perfuses the skin and over a period of 6-7 hr reaches the isotonic saline solution underneath. After this inaugural delay period a steady rate of penetration was maintained and after 24 hr about 1 mg of the complex perfused the skin. Histological examination showed that copper was present in all layers of the perfused skin.[25]

However, the possible acceptance of this form of therapy was impeded by the development of aspirin-like non-steroid anti-inflammatory drugs (NSAIDs), DMARDs and anti-inflammatory steroids (glucocorticoids). In 1960 Bonta[12] showed that copper compounds possessed anti-inflammatory effects in animals and Sorenson[2] confirmed these findings.[14] It was speculated that anti-arthritic agents might promote tissue redistribution of elevated serum copper in RA patients, which was a physiological response to the inflammation.[15] Studies examining the effect of copper on inflammation have recently been the subject of considerable attention.[16] The objective, then, in designing copper based anti-inflammatory drugs, has been to increase the concentration of the low molecular mass, membrane penetrable plasma fraction of copper.[23]

This may be achieved in three ways:

1. Copper may be released from ceruloplasmin or some other inert copper store. This approach would be the most difficult as it would involve chemical degradation of the protein or reductive chelation of the metal.
2. The copper reversibly bound to serum albumin may be removed using a powerful low molecular weight copper complexing ligand.
3. Copper could be administered orally or topically as a neutral, membrane penetrable, low molecular mass complex.

Ultimately, the only way of testing drug efficiency is with animal screens.[17] The increase in serum copper that occurs after copper complex administration is also dependent on the route of administration and the species administered.[16] Furthermore since an elevated serum copper level is a feature of RA, prophylactic effects have been assessed of a variety of drugs of the copper levels. These effects have been correlated with the anti-arthritic activity of the drugs.[18] Presently, a large amount of data indicates that endogenous copper plays an important role in many biochemical processes,[18] some of which are closely connected with inflammation.[19]

Sorenson [20] and Jackson [21] et al have shown that Cu(II) complexes are effective in reducing the inflammation associated with RA. Reports abound in the literature concerning the active role of copper complexes in the control of inflammatory diseases.[22] A great deal is known about the role of copper complexation in enhancing the pharmacological profile of NSAID activity and reducing toxicity.[22] Other pharmacological activities of copper complexes, and their potential as antiarthritic, antiulcer, anticancer drugs have been reported.[22] These activities of copper complexes support the hypothesis that the corresponding disease is, in part, inflammatory, and emphasize the need for more research in the area of essential metal metabolism and inflammation.[22]

2.2 Aim of Study and Objective

Both Cu(II) and diclofenac are used to alleviate the inflammation associated with RA. It has been postulated that a combination of the two agents will have a synergistic effect. That is, their combination would be more effective than when these components are administered independently. If this is true there must be some chemical reason for the synergism and should be related to the thermodynamic stability of the system.

The aim of this investigation then is to test the synergism hypothesis and to elaborate any underlying chemical basis for it by: (i) determining the thermodynamic stability of the Cu(II)-DCL system. (ii) The determined formation constants will be used in computer models of intestinal fluids and blood plasma in order to investigate the speciation of the two components in these biological fluids. In this way we hope to demonstrate the existence or otherwise of the Cu(II)-DCL complex in solution. (iii) UV/Vis spectroscopy will be used to predict the structure of any complexes formed.

2.3 Dissertation Outline

Chapter 1 (Introduction) gives an overview of the nature of rheumatoid arthritis and its therapy. Chapter 2 (Motivational Study) focuses on the effectiveness of the drug diclofenac (as well as its encapsulation in cyclodextrins) and copper activity in the treatment of arthritis. This culminates in formulation of the aim of the study, namely to investigate the hypothesis of Cu-DCL synergy. Chapter 3 deals with theory and practical application of glass electrode potentiometry. Under Experimental Potentiometry sufficient detail of the instrumentation used and the preparation of the solutions is given followed by the description of titrations and data analyses. Results and discussion follow at the end of the chapter. In Chapter 4 (Spectrophotometry) attempts to deduce structural information from absorption spectra and the species distribution of Cu-DCL complexes as a function of pH have been made. The severe problem of insolubility encountered complicated the study but the methods employed to attempt to overcome this (use of cyclodextrin and MeOH/H_2O) were well-founded. The complexes found were then used in computer models of intestinal fluids and blood plasma to calculate the speciation of the two components. Chapter 5 (Conclusion) gives a reasonable summary of the main conclusions and their medicinal significance.

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CHAPTER THREE

Glass Electrode Potentiometry

3.1 *Theory*

3.1.1 Introduction.

The glass electrode is to potentiometry what the silicon chip is to computers - the central component.[1] The glass electrode is dependent upon supporting instrumentation such as voltmeters and reference electrodes. It is equally possible to use glass electrodes to measure hydrogen ion concentrations, expressed as $[H^+]$, or activities, expressed as pH. In 1875 Lord Kelvin suggested that glass was an electrolytic conductor and later in 1906 Cremer reported an Electro-Motive-Force (emf) across a thin glass membrane separating two aqueous solutions and noted that this glass electrode potential was sensitive to changes in acidity. Ideally, glass membranes ought to show a perfect Nernstian response to pH.[1]

It is well known that no glass gives a true response; not only E^o but also the Nernstian slope tend to be variable and so each electrode has to be calibrated either using buffers to read pH or using standard acid and alkali solutions to read $[H^+]$. Both the glass and reference electrodes require filling solutions, which over the decades potassium chloride (KCl) has evolved as the usual first choice because it seems to satisfy most of the criteria, especially that of the diffusion rates of anion and cation being equal.

pH measurement using an electrochemical cell, therefore, requires two electrodes,[1] one being the working hydrogen - ion activity- sensitive electrode and the other a hydrogen- ion activity- insensitive electrode. There is a reference electrode that embodies an internal electrode such as Ag/AgCl cell (Ag/AgCl electrode being inside the glass membrane and provides a constant potential[2]) and an electrolytic solution contained in a glass/ polymer salt bridge, which surrounds the internal electrodes and makes electrical contact with the test solution through the liquid junction. The only potential which can vary is that existing between the outer surface of the glass bulb and the test solution in which it is immersed, and so the overall potential of the electrode is governed by the hydrogen ion concentration of the test solution.

To measure the hydrogen ion concentration of a solution the glass electrode must be combined with a reference electrode, for which purpose the saturated calomel electrode is most commonly used, thus giving the cell:[2]

Ag, AgCl(s)/HCl (0.1M)/ Glass/Test soln | | KCl (Sat'd); Hg_2Cl_2(s)/Hg

3.1.2 Potentiometric Titration

Potentiometry[3] is one of the most convenient and successful techniques employed by metal complex equilibrium measurements. With metal electrodes it is usually sufficient to use the highly accurate glass electrode for measuring the hydrogen ion concentration in a procedure termed potentiometric titration. In this, for example, standard base is added in increments to a well characterised acid solution of the ligand in the absence of and in the presence of known metal ion concentrations. While there are many other methods for measuring equilibrium constants e.g. ion exchange, NMR, polarography etc, their use is usually invoked under special circumstances, which arise when potentiometry or spectrophotometry cannot be employed. Potentiometry does not provide microscopic information involving identification of protonation and metal coordination sites on a ligand. For such information spectroscopic measurement or spectrophotometric absorbance studies are needed.[3]

As we have said earlier, this procedure is concerned with changes in electrode potential rather than in an accurate value for the electrode potential with a given solution, and under these circumstances the effect of the liquid junction potential may be ignored.[2] The change in cell emf occurs most rapidly in the neighbourhood of the end points.

Generally speaking the end point of a titration can be most easily fixed by examination of the titration curve including the derivative curves to which this gives rise or by examining a Gran's plot.[2]

3.1.3 Titrimetry Analysis

This refers to quantitative chemical analysis carried out by determining the volume of a solution of accurately known concentration which is required to react quantitatively with a measured volume of a solution of the substance to be determined. When measurements are made the filling hole of the electrode must be left open to the atmosphere; otherwise the flow of liquid through the liquid junction will stop.[1] A slow leakage through the junction is needed in order to maintain electrical contact with the surrounding solution.

The liquid junction is an important feature of the reference electrode. For storage periods of long duration the glass electrode should be stored dry and before using it again it must be soaked.[1] The reference electrode should also be stored dry and sealed or with its tip immersed in a salt- bridge solution. For short-term storage, i.e. between titrations, or overnight, it is important to keep the surface layer on the glass intact. Electrodes that are not customarily taken to the acid end of the pH scale should be dipped occasionally in mildly acid solution in order to decrease the response time.

Reference electrodes should not be stored in distilled water or weak solutions as this can lead to dilution of the filling solution. Whenever the electrode is stored for more than an hour or so, it is important to ensure that it is placed in a vessel in which the electrode stem and vessel are closely joined. This prevents evaporation of the storage solution and thus prevents the formation of a crust of salt on a tip of the electrode.[1] Electrodes are invariably washed as part of the transference procedure from one solution to another. This is conveniently done by giving them three large drenchings with deionised water and then blotting them dry with tissues. Water used in all electrode manipulations and solutions should be of good quality and glass distilled.[1]

When a metal M is immersed in a solution containing its own ions M^{n+} then an electrode potential is established the value of which is given by the Nernst equation:[2]

$$E = E^{\theta} + (RT/nF) \log a_{m}^{n+}$$

where E^{θ} is a constant, the standard electrode potential of the metal. E can be measured by combining the electrode with the reference electrode and measuring the emf of the resultant cell.

It follows that knowing the potential E_r of the reference electrode, we can deduce the value of the electrode potential E and provided the standard electrode potential E_θ of the given metal is known, we can then proceed to calculate the metal ion activity a_m^{n+} in the solution. For a dilute solution the measured ionic activity will be virtually the same as the ionic concentration and for stronger solutions, given the value of the activity coefficient, we can convert the measured ionic activity into the corresponding concentration.[2]

3.1.4 Reaction Mixture

The reaction solution is made up in a cell having features as the glass electrode to hydrogen ion activity, a reference electrode that is intended to be the constant potential.[4] The temperature is controlled by circulation of thermostated water through the jacket. The solution must be completely sealed from the atmosphere through the use of O-rings. The cap may be machined plastic with grooves for the O-rings seals. The inert gas must be purified to remove CO_2 and O_2 and humidified with a background electrolyte. The standard base/ acid is added through a capillary tip beneath the surface of the solution and is measured by a piston-type burette capable of reading volumes down to 0.01 ml or better.

There should be a sufficient number of openings in the cap to take care of the electrodes and all materials introduced into the reaction mixture. The reaction solution can be made up by adding precisely measured volumes of ligand solution, standard acid if needed, metal ion solution when appropriate. Sufficient solid supporting electrolyte must also be added to provide the ionic strength desired for the electrolyte, metal salt and ligand contributions to the ionic strength (I).[4] There are several salts used to keep the I of a test solution constant. The kind of salt selected for the purpose must such that it dissolves to yield ions that are common to the reacting acid and base, so as to eliminate the introduction of other factors of variation.[1] Doubly distilled water may then be added to make up the volume exactly to the pre-determined amount.[4]

3.1.5 Calibration of the Electrode

Prior to making measurements on the experimental solutions, it is necessary to calibrate the pH meter and electrode system in terms of hydrogen ion concentration.[3] The glass electrode has an asymmetry potential which makes it impossible to relate a measured electrode potential directly to the pH of the solution and makes it necessary to calibrate the electrode.[2] The slope of the electrode is determined using standard buffers.

The Nernst equation shows that the glass electrode potential for the given pH values will be dependent upon the temperature of the solution. In this case a buffer line is obtained by plotting the observed emf for each buffer against the pH.[5] The slope of this line gives the value of s, for the electrode:

$$S = E_{4.01} - E_{9.18}/9.18-4.01$$

The acceptable working range of the electrode response slope at 25°C is: $58.60 < s < 59.16$

This method is adequate if only relative measures of the hydrogen ion concentration are required.[5] More precise calibration may be achieved by plotting the hydrogen ion concentration observed at each point of a strong acid – strong base titration performed at constant ionic strength against the observed emf. The most suitable pH regions for glass electrode calibration are 2.3 to 2.9 in the acid region and 10.8 to 11.3 in the alkaline region.[5]

3.1.6 The Potentiometric Cell

Immersed in the test solution are two "electrodes", more precisely described as the probes for the species being monitored and a liquid junction J leading to a reference half-cell via a salt bridge. [6] The cell can be written as:

-reference half-cell | salt bridge | test solution | 1
j J

The Nernst equation for electrode response can then be expressed in terms of concentrations rather than activities. The emf of cell 1 is given by:

$$E_{cell} = E_p - E_{ref} + E_J + E_j \quad \text{.....2}$$

where E_p and E_{ref} are respectively the potentials of the probe and reference half cells, and E_J and Ej are the diffusion potentials at junctions J and j. Many probes respond to the concentration of a single species S according to the relationship: [6]

$E_p = E_p^\circ + \lambda_s RTF^{-1} \ln [S]$ 3

where E_p° and λ_s are constants. Since values of E_{ref} and E_j are unaffected by changes in the test solution, the emf of cell 1 can be written as:

$E_{cell} = E^\circ + \lambda_s RTF^{-1} \ln [S] + E_J$ 4

where the value of

$E^\circ = E_p^\circ + E_{ref} + E_j$ 5

is constant. It is often convenient to combine E° and E_J to give

$E^{\circ\prime}_{const} = E^\circ + E_J$ 6

which will also be constant provided that E_J does not vary. If this condition is fulfilled equation 4 becomes

$E_{cell} = E^{\circ\prime}_{const} + \lambda_S RTF^{-1} \ln [S]$

The temperature of the cell has to be kept constant so that the slope $\lambda_S RTF^{-1}$ remains constant.[5]

3.1.7 Ligand Reaction Equations.

Interest[7] in the determination of stability constants of metal ion complexes was greatly stimulated by the work of J.Bjerrum who elaborated a general method for the determination and calculation of the stability constants for metal ammine complexes. It was shown that unidentate ligands invariably are added in a succession of steps, which can be represented as:[7]

$ML_{n-1} + L \leftrightarrow ML_n$

in which[8] the species ML_n is formed by the addition of one ligand to the preceding complex ML_{n-1}.

Similarly, in the usual case, where the preceding complex ML_{n-1} can also exist, the activity of ML_n can be expressed in terms of the activities of ML_{n-1} and L.[9] Thus:

$\{ML_n\} = {}^T\beta_n \{M\}\{L\}^n = {}^TK_n\{ML_{n-1}\}\{A\}$

where the overall stability constant ${}^T\beta_n$ is the activity quotient for the reaction step

$M + nL \leftrightarrow ML_n$

Thus ${}^T\beta_o = {}^TK_o = 1$ and ${}^T\beta_1 = {}^TK_1$

Moreover, the overall and step stability constants are related by the expression[9]

$$\beta_n = K_1K_2K_3 \ldots K_n = \prod_1^n K_n$$

The numerical values of the stability constants of a given complex depend not only on the experimental conditions employed but also on the units in which the activity terms are expressed.[9]

$\prod_1^n K_n$ indicates[10] the product of all the stepwise stability constants up to the n^{th} step. In the absence of the metal ions, the protonation constants of a ligand in the chemical equation: $L + rH \leftrightarrow LH_r$

May be expressed as the overall stability constant: $\beta = [LH_r]/[L][H]^r$

or as in the stepwise formation:

$LH_{r-1} + H \leftrightarrow LH_r$

As the stepwise stability constant:

$K = [LH_r]/[LH_{r-1}][H]$

The relationship between these two kinds of constants is:

$$\beta = \prod_1^r K_{H,i}$$

indicating the accumulation of the stepwise stability constants, where $K_{H,i}$ is the constant of the protonation of the ligand by the ith proton.[10]

3.1.8 The Ionic Strength.

The concept[9] of ionic strength was introduced in 1921 by Lewis and Randall, who stated that in dilute solutions, the activity coefficient of a given strong electrolyte is the same in all solutions of the same ionic strength. A theoretical relationship between the activity coefficient and the ionic strength was derived by Debye and Huckel in 1923, and since then many workers have attempted to keep activity coefficients constant by using solutions of constant ionic strength.[9]

Although it is sufficiently accurate at low ionic strength to choose some form of the extended Debye-Huckel equation for the calculation of activity coefficients, it is better at higher concentrations to determine the most appropriate parameter values.[11] For ions it also varies with the ionic charge, and is the same for all dilute solutions having the same ionic strength, the latter being a measure of an electrical field existing in the solution. The term ionic strength designated by the symbol I, is defined as equal to one half of the sum of the products of the concentration of each ion multiplied by the square of each charge number or: $I = 0.5 \sum c_i Z_i^2$ where c_i is the ionic concentration in mole per litre of solution and Z_i is the charge number of the ion concerned.

It can be shown on the basis of the Debye-Huckel theory that for aqueous solutions at room temperatures:

$\text{Log } Y_i = -0.509\ Zi^2\ (I/\text{molkg}^{-1})^{0.5}$

where Y_i is the activity coefficient of the ion; Z_i is the charge of the ion concerned, I is the ionic strength of the solution.[37]

3.1.9 Stability Constants.

A large and very important group of equilibria is that which involve the formation of complexes between a metal ion and various Lewis bases known as ligands. A wide variety of anions and molecules can act as ligands usually by donating an electron pair to the metal ion to form a sigma bond.[8]

Although almost any metal ion, given a suitable ligand, can act as the central group of a complex, some form complexes more readily than others. In aqueous solution a metal ion becomes surrounded by a number of other ions or small neutral molecules, for example, water molecules, in a fairly permanent association.[12]

The aggregate consisting of a metal ion together with its collection of followers is called a metal ion. Following Rossotti & Rossotti,[13] a complex can be defined as a species formed by association of two or more simple species, each normally capable of independent existence.

The stability of a complex may be expressed quantitatively in terms of one of its stability constants.[9] If the two species M and L coexist in solution, they may react to form one or more complexes of general formula ML_n where the species M comprises the central atom to which the species L, usually referred to as a Ligand, is coordinated. We can generalize these reactions for simplicity as follows:[13]

$M + nL \leftrightarrow ML_r$ 1

Polynuclear complexes[13], MpLqHr (for which p >1, q ≥0, r ≥0), are frequently encountered in aqueous solution chemistry, particularly in hydrolysis reactions where $L = OH^{-1}$. The ligand L may be monodentate, bidentate or polydentate, i.e., it may coordinate using one, two or more donor atoms.[9] Ions of metals towards the end of the d-transition series form a wide variety of stable complexes with ligands, which contain nitrogen atoms. Some ligands contain more than one donor atom, and so can be attached to the metal ion at more than one binding site, for example glycinate ion (gly^-).[8]

They act as chelating agents, with two donor atoms clipping on to the metal ion like the two pincers of a crab's claw. The number of ligands which can bind a central metal ion naturally depends on the number of donor atoms which are used by each ligand group and by the preferred stereochemistry and relative sizes of both ligand and metal ion.[8] Stability constants, or equilibrium constants for metal complex formation, have long been employed as an effective measure of the affinity of a ligand for metal ions in a solution, and have served as a quantitative indication of the success or failure of the ligand design.[3]

By 1953, stability constants for a number of metal complexes had been reported and it was feasible to ask the following questions:[13]

1. for a given ligand, how does the stability constant vary with changes in the central metal ion?
2. how does the stability vary when the nature of the coordinating ligand, L, is changed?

After reviewing all the data, Irving and Williams showed that for divalent ions of the first transition series the stability always follows the order: Mn <Fe <Co <Ni <Cu <Zn. For each cation the stability increased when donor oxygen was replaced by donor nitrogen in either monodentate or bidentate ligands.

As the number of chelated rings increased so the stability increased, but it decreased with increase in ring size. The order of decreasing stability $F^- \gg Cl^- > Br^- > I^-$ was followed by most cations (Group (i)), but was reversed for a few elements such as Ag^+, Cu^+, Hg^{2+}, Tl^{3+} for which the stability increased in the order $F^- < Cl^- < Br^- < I^-$.[13]

3.2 Copper-Glycine Complex.

3.2.1 Introduction.

Glycine ($H_2N\text{-}CH_2\text{-}COOH$) is the simplest α-amino carboxylic acid for which the side chain is H.[17] But it never occurs to an appreciable extent in this form.[14] Like all the amino acids in their pure states, it exists as a dipolar ion, a zwitterionic form being more favoured energetically both in solution and in the solid state.[14] Glycinate ion (gly^-) is a bidentate ligand (literally two teeth),[15] because it bonds to a metal using an electron (e^-) pair on each of the nitrogen and the oxygen atom. See Figure 2 below:[16]

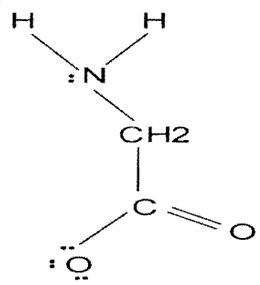
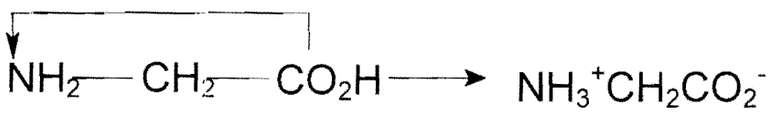


Figure 2 Glycinate ion (gly^-)



Amino acetic acid (glycine)>>>>>>>>>>>>>glycine dipolar ionic form

This ligand can bond two different donor atoms to the one metal atom and this can lead to unsymmetrical arrangements of ligand groups about the metal ion (see Figure 3).[15]

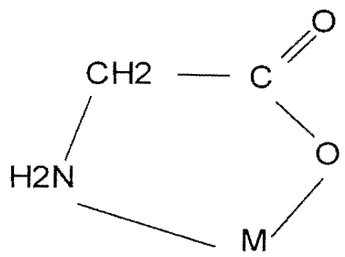


Figure 3. The glycinato chelate ring

The fully protonated glycine cation, $[H_2L]^+$, contains two ionisable H ions, which dissociate stepwise in fully separated processes. Depending on the pH of is solution, glycine can exist in three different forms: the cationic $[H_2L]^+$, the zwitterionic $[HL]^{\pm}$ and the anionic L^- forms. Since the dissociation steps are well separated, the concentration of the neutral form HL is negligible. Besides these monomeric proton complexes, various polymeric associates e.g. $[H_2L]^-$, H_2L_2 and $[H_3L_2]^+$, can also be formed in concentrated solution.[15] The main reason for the association of the monomeric species is the formation of H-bonds between them, whereas in dilute aqueous solution the monomeric species are stabilized through H-bonds with the water molecules. [15]

3.2.2 Presentation of equilibrium data.

The proton complex formation constants of glycine are expressed as stepwise protonation constants. For the equilibria

$H^+ + L^- = HL$ (protonation of $-NH_2$ group) and.....3

$H^+ + HL = H_2L$ (protonation of $-COO^-$ group) 4

the constant K_1 relates to the first processes and K_2 to the second:

$$K_1 = \frac{[HL]}{[H^+][L^-]}$$

3.2.3 Metal complex stability constants.

$Cu(AA)_n$ complexes (AA = amino acid) have already been the subject of detailed investigations in solution.[18] Complexation constants of different AA with Cu^{2+} were determined.[19,20] The type of binding between an AA and Cu^{2+} is the same for all α-amino acids with the exception of histidine.[21] Of the metal complexes, those of the 3d transition metal ion especially Cu(II) have been studied in most detail.[14]

In order to validate the experimental procedure and method, this system was studied before moving onto the Cu-diclofenac system

3.3 Experimental Potentiometry

3.3.1 Instrumentation and Chemicals.

Potentiometer	PHM 84 RESEARCH pH METER
Burette	DOSIMAT 665 Automatic Burette
Electrodes	Ω Metrohm 6.0133.100 & 6.0726.100 (ref.)
	pH 0 . . . 14 / 0 . . . 80°C
	Ref. Ag, AgCl, 3M KCl
Acid	0.1M HCl
Base	0.1M NaOH
Background Electrolyte	0.15M NaCl

All the titrations were automatically controlled. The electrometric cell in which the metal-ligand interactions were studied consisted of a jacketed beaker equipped with magnetic stirrer and fitted with a tight-fitting rubber stopper through which were inserted an inlet and an outlet tube for nitrogen gas, a glass and a reference extension electrode. There is also an opening for the materials introduced in the reaction vessel like the addition of the standardised acid and base for titration in the solution.

The magnetic stirrer that was used allows a homogenous solution to be obtained giving a fairly fast response time but all calibration procedures were carried out at the same stirring rates.

Care should be taken to ensure that the stirrer does not heat the solution. Also, the magnetic follower does not bump when rotating and thereby damage the electrodes.

3.3.2 Preparation of Solutions.

The experimental solutions in the cell were each maintained constant at 25°C by the circulation of the thermostated water from a mercury contact thermoregulator controlled water bath through the jacketed cell. The ionic strength of all solutions were maintained at 0.15M using NaCl as the background electrolyte.

3.3.2.1 Acid Solution (HCl)

These were made from 0.1M BDH CVX ampoules on the 500ml volumetric flask. After preparation was complete standardisation against recrystallised borax was done.

3.3.2.2 Base Solution (NaOH).

NaOH solutions were also made from BDH CONVOL ampoules in a 500ml volumetric flask to make a 0.1M solution. The presence of carbonate in the NaOH was checked using the method of May *et al.*[36] Because the solution must be CO_2 free N_2 gas was pumped through the solution before it was stored in high-density polypropylene bottles.

Standardisation of the base solution was carried out against recrystallized KHP (potassium hydrogen phosphate).

3.3.2.3 Solutions of ligands.

Solutions of glycine and diclofenac were freshly prepared using high purity samples obtained from Sigma –Aldrich Co. and they were standardised potentiometrically. For each titration the required amounts of the ligand were weighed out and were allowed to dissolve in the experimental cell under N_2 atmosphere.

3.3.2.4 Solution of the Cu (II) Metal ion.

Stock solution of Cu (II) ion was prepared by dissolving the required quantities of the respective chlorides (reagent grade) in double distilled water and standardised by complexometric titrations.

3.3.3 Calibration of Electrodes.

Prior to calibration the electrode was stored overnight in a 0.1M HCl solution. It was repeatedly rinsed with distilled water and patted dry with soft tissue paper. It was blotted dry and not wiped in order to minimise the build up of static electricity. The slope of the electrode was checked using standard buffers. The emf reading at 25°C was recorded and was used to calculate E°, the glass electrode potential.

3.3.4 Titrations.

3.3.4.1 Glycine Protonation Titration.

An approximate amount of the background electrolyte NaCl solution was dispensed into the titration vessel having the weighed out ligand followed by small addition of the required concentration of the standardised HCl solution from another burette. This volume of HCl solution was added to the vessel to ensure that the ligand was completely protonated. The electrodes completed the reaction cell and the solution was allowed to stand until the working temperature had been attained and all the ligand had dissolved.

Potentiometric titration was followed with the glass electrode at base increments of NaOH. Data obtained consisted of the volume of base added and the corresponding emf values of the system at each titration point. This was used in the data analyses discussed below.

3.3.4.2 Diclofenac protonation Constants Titration.

Diclofenac is insoluble in water and precipitates at low pH values, so the sodium salt was titrated from high to low pH until precipitation occurred. The insolubility of diclofenac forced us to employ two titration methods for this particular ligand.

Previous work reported that diclofenac is highly soluble in organic solvents including methanol so a 50% v/v solution of methanol in water was used in one method to dissolve the ligand. Another method employed was β-CD, which has the ability to encapsulate drug molecules. In these titrations β-CD and diclofenac were weighed directly into the titration vessel to give 20ml of a 0.01M and approximately 8 x 10^{-4} M solution of β-CD and diclofenac respectively. Both titrations for the two methods used were carried out with increments of HCl.

It has been observed that in the range between 8.2 and 12.7 pH units, the electrodes' response is practically constant and independent of pH because diclofenac was completely ionised in this pH zone. In the pH zones <8.2, there was a precipitation of diclofenac in the form of acid, by protonation of the secondary amine. Also the interference from the H^+ ion causes an oscillation in potential.

Considering this behaviour an acidic solution was employed throughout the investigation process. For diclofenac it became necessary to find E° and pKw for 50% methanol as a solvent.

3.3.5 Data Analysis.

For glycine, the concentrations of both acid and ligand were used as input into an ESTA file template for the determination of the protonation constants. ESTA is used for simulating simple equilibrium distributions of chemical species. The major objective in ESTA is to provide a flexible tool for investigating phenomena associated with chemical interactions in solution and for their quantitative characteristics.[36] Also ESTA program being the refinement of stability constants from potentiometric data measured on protonation and complexation systems containing any number of interacting components. Whilst the situation is different for diclofenac, the concentration of ligand was then used as input into an ESTA file template for the determination of the protonation constants.

These results were then used to generate Z_H curves. It should be noted that, before any good agreement obtained, several adjustments to experimental concentrations were necessary. Several different titrations were performed, each with slightly different initial concentrations of ligand.

3.3.5.1 Metal ion Complexation Titration.

These were also done under an inert N_2 atmosphere where the mole ratio of the metal to ligand was varied. For diclofenac, weighed amounts of the ligand were put in the reaction vessel together with β-CD, Cu^{2+} and NaCl for one method.

For the second method, weighed amounts of diclofenac dissolved in 50% MeOH/H_2O and then addition of Cu^{2+} were put in the reaction vessel and the titration for metal ion complexation run with increments of acid. The water used to make the 50% MeOH/H_2O solution had an ionic strength of 0.15M (NaCl). For glycine, although enough acid was added to raise the pH to the relevant pH range the initial volume in the reaction vessel for glycine was held constant for all of the experiments. Also the metal ion was added from another burette in small ranges and the titration proceeded by base increments of NaOH.

3.3.5.2 Complexometric Titration Data Analysis.

The titration data for each of the pairs of titrations carried out at different metal: ligand ratios were first analysed separately as pairs and then all together as a whole. In each case the input data i.e. protonation constants determined as outlined above, the calculated concentrations of the metal, ligand and hydrogen ion were included. Also the electrode potential calculated from a strong acid reading taken immediately prior to the titration and the dissociation constants of water and methanol was included.

Even with this task the calculated and observed plots must completely overlap if the model is correct. It should be said that the accuracy that is needed for potentiometric work is a skill that is acquired with wider exposure to and practice in the technique.

The results in the following section are from sets of repetitive experiments.

3.4 Results.

In order to validate the experimental procedure, the known system of Cu/glycine was studied and results compared to literature (Table 3)

Table 3.The logarithms of the stability constants measured for the Cu(II) glycinate system MpLqHr at 25°C, I =0.15 M Rf = Hamilton R factor, Rlim = Hamilton R limit, σ= standard deviation, n = number of points

p	q	r	β_{pqr} (lit)[19]	β_{pqr} (exp)	σ	R_f	R_{lim}	n
0	1	1	9.65	9.65	0.002	0.002	0.001	101
0	1	2	11.86	11.86	0.002			
1	1	0	8.20	8.17	0.003	0.003	0.001	230
1	2	0	15.07	14.95	0.003			

[19]*Stability constants measured at 25°C, I =0.15 M KCl*

Table 4.The logarithms of the stability constants measured for the Cu(II)-DCL system MpLqHr at 25°C, I =0.15 M. βCD = β-Cyclodextrin, Met = Methanol, Rf = Hamilton R factor, Rlim = Hamilton R limit σ = standard deviation, n = number of points

Conditions	p	q	r	β_{pqr} (exp)	σ	R_f	R_{lim}	n
0.1 M βCD	0	1	1	4.29	0.005	0.03	0.03	54
50% Met/H_2O	0	1	1	4.82	0.002	0.01	0.01	731
	1	1	0	3.48	0.051	0.01	0.01	128
	1	1	-1	-3.02	0.068			

3.5 Discussion.

The protonation and metal ZBAR curves for glycine are given in Figure 4 and 5 respectively. As can be seen there is a good agreement between titrations under different conditions. For glycine, a statistical analysis of the results indicates that there is a good fit between the theoretical model and the experimental data (see Table 3 above). In addition there is a good agreement with the literature. The largest error is 0.12 log units which is acceptable.

Figure 4. ZBAR curves for glycine titrations at 25° C and I=0.15M(NaCl). The total conc. Of Glycine is indicated.

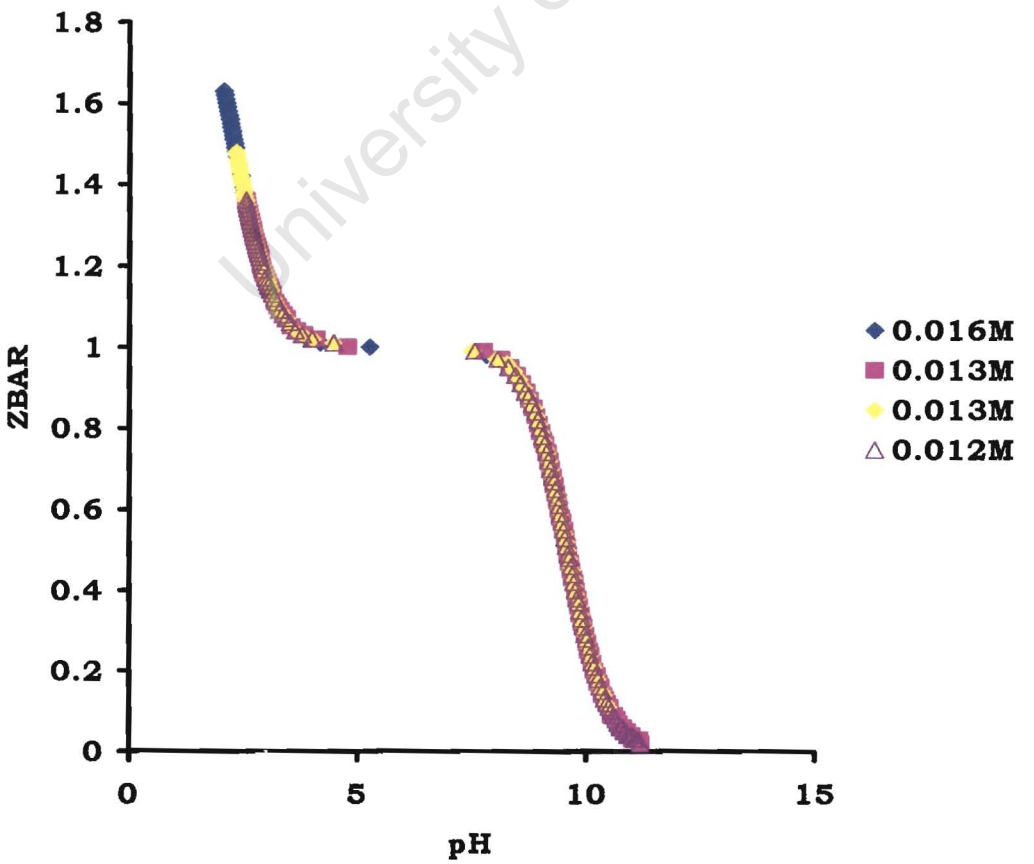
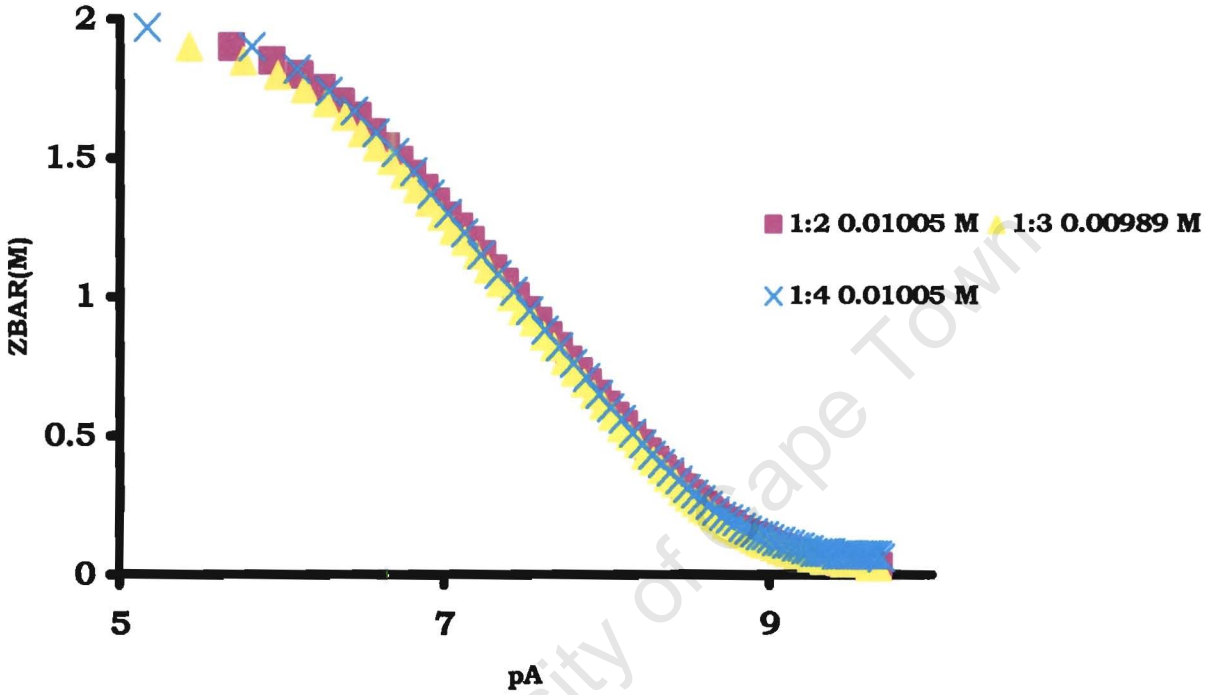
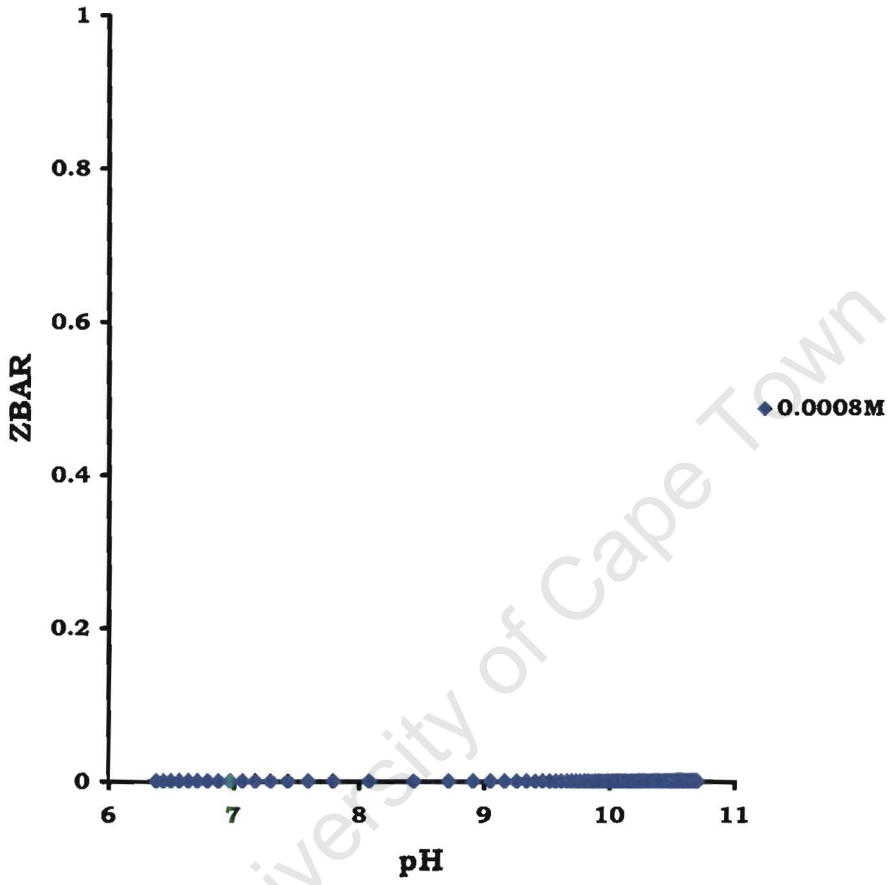


Figure 5. ZBAR curves for Cu-Glycine titrations at 25° C and I =0.15M (NaCl). The ratio of M:L ratio given with total conc. of Gly



Potentiometric measurements were performed for the determination of the protonation constant of sodium diclofenac and complexation of the ligand. As DCL is insoluble in acid medium, the normal procedure of titrating with base from low to high pH could not be used. Instead, the ligand was dissolved in base and then titrated with acid. As soon as precipitation was observable or the electrode response became unstable the titration was stopped. Generally the electrode response became unstable before precipitation could be clearly seen. A ZBAR plot for these titrations is shown in Figure 6 below.

Figure 6. ZBAR curves for Diclofenac (0.0008M) titrations at 25° C and I = 0.15M (NaCl)

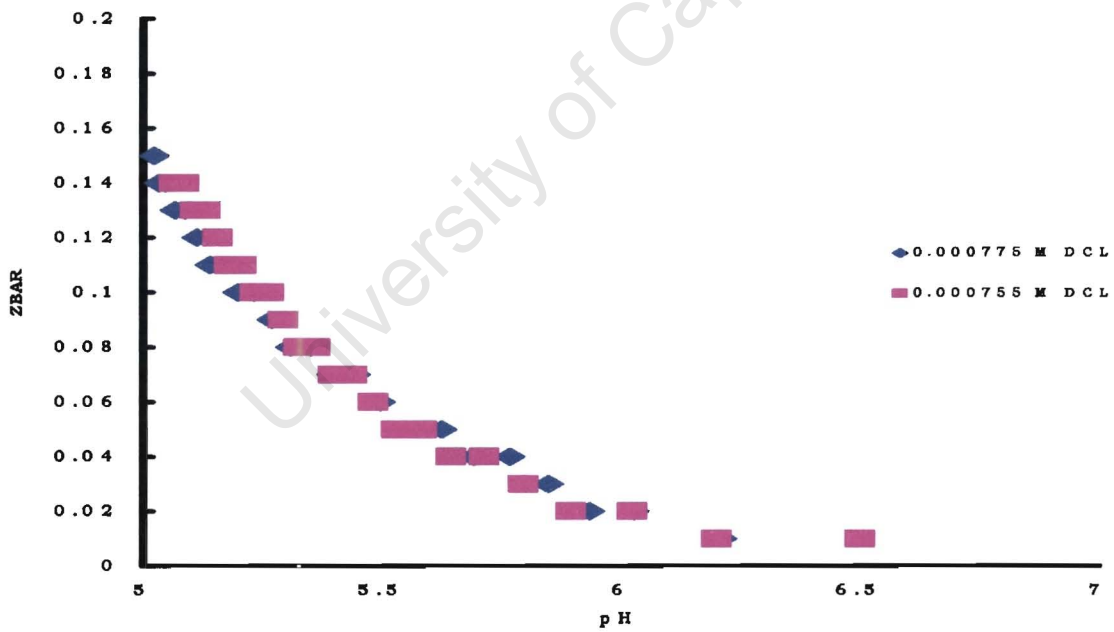


As can be seen the maximum ZBAR value was 0, which is too low for a meaningful determination of its protonation constant. One possible way of overcoming the problem of precipitation was to work at lower concentrations. However, the final concentration was 8 $x10^{-4}$M, which was near the limit for our experimental set-up.

Because of the difficulties of precipitation the experiments were repeated using β-CD as a carrier molecule.

Microencapsulation of drug molecules in CDs has been used extensively in the pharmaceutical industry to produce more soluble drug preparations with improved bioavailability. The results for the titration of DCL in the presence of β-CD are given in Figure 7. These results show that protonation of DCL is indeed occurring and a ZBAR value of 0.15 is attained before precipitation occurs. This happens at pH ~5.

Figure 7. ZBAR curves for βCD (0.01M)-DCL titrations at 25°C and I = 0.15M (NaCl), the conc. of DCL given.



A 100-fold excess of β-CD was used so that, under these conditions, the DCL was assumed to be fully encapsulated. The results of the ESTA analysis of the data are given in Table 4, a value of 4.3 being obtained for log β_{011}.

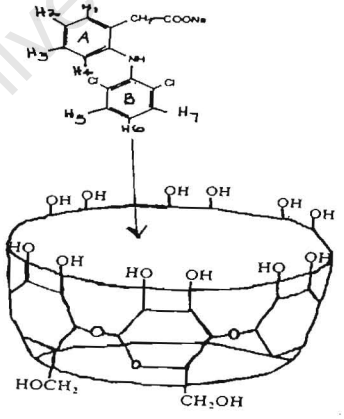
This result is different to that obtained by J.A Arancibia[27] in his studies where a value of 4.9 was obtained under the same conditions. However the value of 4.3 seems more reasonable when compared to literature values of analogous compounds such as phenyl acetic acid (pKa =4.1, t=25°C and μ= 0.1M)[24] and also acetic acid (pKa =4.53, t=37°C and μ= 0.15M). J.A Arancibia[27] also managed to measure an equilibrium constant of $10^{4.8}$ for DCL in aqueous solution but under their conditions precipitation was observed by us.

The agreement between the results in β-CD and pure water led J.A Arancibia[27] to postulate that the dichlorophenyl end of the DCL is really included in the β-CD cavity and that the carboxylate group does not interact with β-CD cavity.

However crystal structure of this inclusion complex does not support this conclusion.[29] These studies show interactions in both hydrogen bonding and hydrophobic interactions between the phenylacetic residue of DCL anion and β-CD. Hence the phenyl ring is fully inserted in the β-CD cavity while one carboxylate O_2 atom is hydrogen bonded to a primary OH group of the β-CD molecule. In the crystal structure, the bulky dichlorophenyl group is not in the β-CD cavity but it lies just outside the cavity.[29]

The Cl substituents are shifted away from the middle of β-CD where they can no longer fit inside it and therefore protrude slightly.[34] Also, E.J.Moyo[28] in her studies postulated that the ligand can go into the cavity from the wider side with the phenyl ring (B) going in partly to leave the Cl atoms just standing out (see Figure 8 below). It has been reported that the ionised forms of the drug molecules are both more hydrophilic and carry a larger number of the tightly bound water molecules than the unionized forms.[33] The water molecules are located outside the cyclodextrin cavity, which stabilises the crystal structure. This alone also justifies that it is hard for the chlorine atoms to enter the narrow and relatively lipophilic CD cavity.[33]

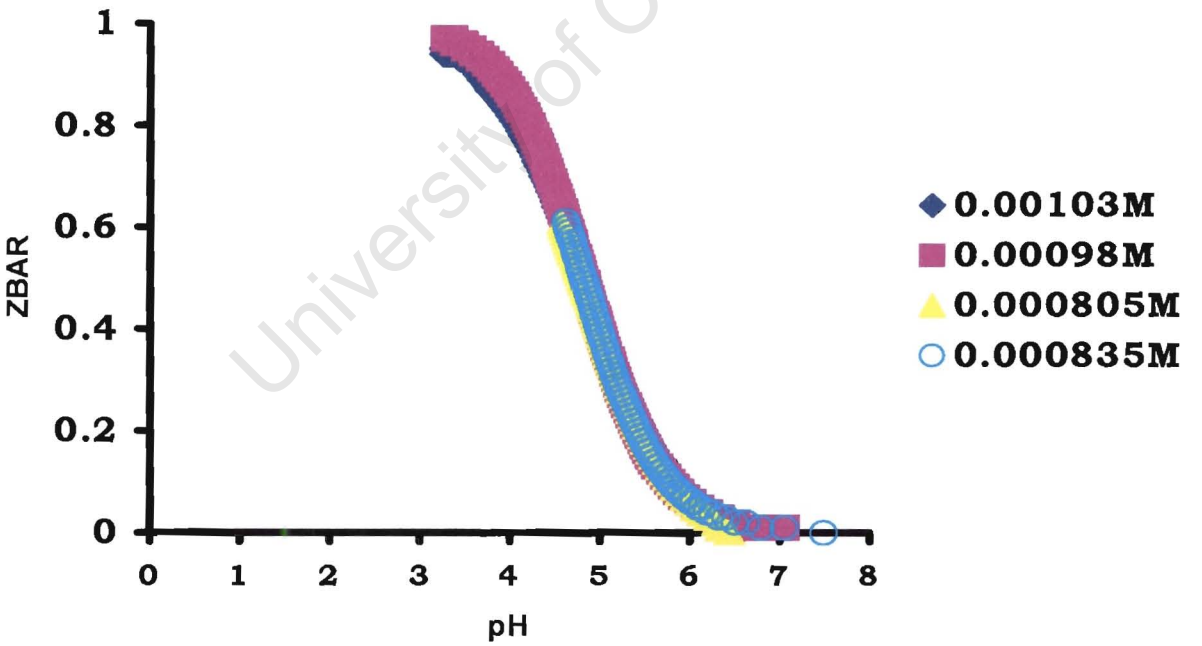
Figure 8. Cyclodextrin inclusion of Diclofenac



But the problem encountered when β-CD was used, was the working pH range, which was only pH 5-7. Below pH 4.5 the electrode potential becomes unstable due to precipitation.

Because of this, another method of improving the solubility of the ligand was tried. DCL is highly soluble in organic medium so a 50% MeOH\H_2O was used as the solvent. In this medium titrations could be perfomed in the pH range 3-6 before precipitation occurred. Figure 9 below shows the ZBAR plot for this system with the protonation constant given in Table 4. Under these conditions, reproducible titrations were possible with a ZBAR value of 1 being attained.

Figure 9. ZBAR curves for DCL in 50%MeOH\H_2O at 25°C and I = 0.15M NaCl, the conc. of DCL given.



The results in Table 4 show that the Hamilton R_f values and standard deviation in 50% MeOH\H_2O are quite low. Hamilton R_f is an estimate of the goodness of fit between experimental emf and the calculated emf refined from the set of stability constants given in Table 4.

The Hamilton R_{lim} is the lowest possible R-value that is statistically significant given the random errors in the experiment. The agreement between R_{lim} and R_f indicates that the model cannot be statistically improved. As can be seen the reproducibility of the titrations were excellent and indicate a well-behaved system.

At 50% MeOH\H_2O, a pKa value of 4.82 was obtained for the carboxylic acid. Similarly, C. J. Byrne[31] and R. G. Bates[32], obtained protonation constant values of 5.47 and 5.54 for phenylacetic acid and acetic acid respectively under the same conditions. These values are slightly higher than the 4.82 obtained for DCL in this study. The most likely reason for this sequence of the stability: acetic acid> phenylacetic acid> DCL, is the inductive effect of the phenyl ring.

Although NSAIDs are very widely used, they are well known for causing gastrointestinal ulceration and bleeding. So complexation of these drugs with CDs was a possible way of reducing irritation by promoting more rapid absorption and therefore shorter exposure to the drug.[34] But because of the smaller working pH zone (pH<4.5) the complexation titrations were not possible with β-CD medium as the ligand just precipitated out of solution.

The titrations for the complexation of the metal ion were then carried out in methanol medium. The experiments were carried out using acid increments as investigated earlier so at approximately pH 6.2 the ligand is fully protonated. But at pH ~5.7 one proton from the protonated carboxylic group is released into the solution due to complexation and a 1 1 0 species formed.

Below pH 5.7 as seen in Figure 11 below, this formed species accumulates in the solution and a slight levelling off of the plot up to pH 4.9 shows this accumulation. At pH 3.9 another species is formed due to the deprotonation of the 1 1 0 species, which is indicated in Figure 10 below by the fanning back of the curve. After pH 3.1 the ligand precipitated out of solution as shown by the sudden drop /fall in of the plot as seen in QBAR curves and all the titrations done were terminated in this region.

Since the ligand has no other dissociation protons deprotonation must be occurring at one of the coordinated water molecules. However the pKa for formation of this species is 6.5 ($\log\beta_{11\text{-}1} - \log\beta 110$), which is much less than the pKa of the free metal (8.1).[35]

Figure 10. ZBAR curves for Cu(0.001M)-MetDCL at 25°C and I = 0.15M (NaCl), the conc. of DCL given.

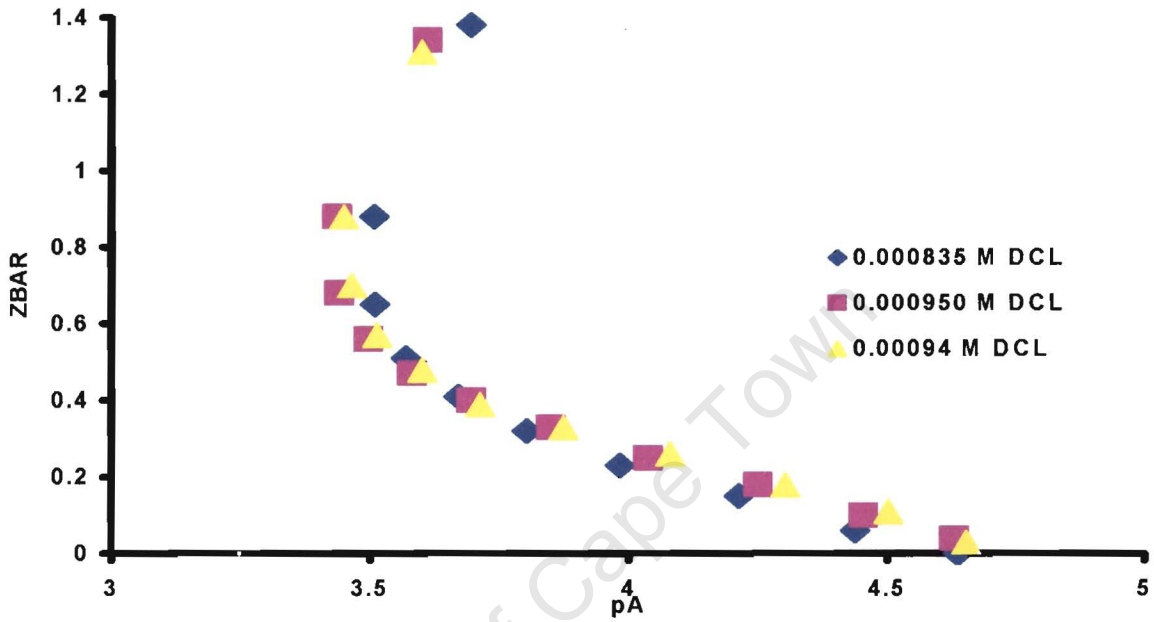
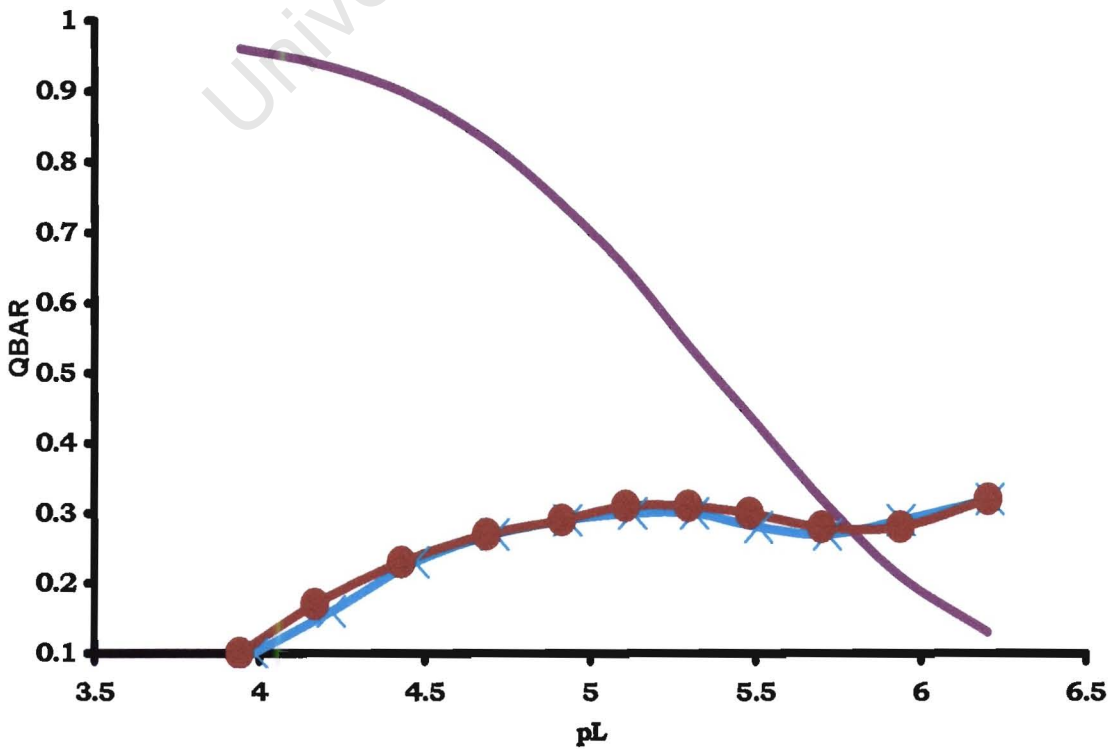


Figure11. Both QBAR and n-bar (boldline) curves for Cu-MetDCL titrations at 25°C and I = 0.15M (NaCl) plotted against pH.



The results of the ESTA analysis are shown in Table 4. A value of 3.5 is obtained for log β_{110} and it can be compared to a value of a log β_{110} 2.7 obtained for Cu/acetic acid in 50% MEOH and also a log β_{110} 2.59 for Cu/phenylacetic acid under the same conditions.[30] The CuDCL complex is a factor of 10 x more stable than the analogous carboxylic acids.

The difference in stability cannot be explained by a difference in the basicity of the acid as all three acids have very similar pKa's. In fact acetic and phenylacetic acid have higher pKa's. We would therefore postulate that the increased stability of the copper complex of DCL results from an involvement of the amine group. Metal ion coordination to both the carboxylate oxygen and the amine would result in a seven membered chelate ring. This would increase the stability of the complex. However because of the two phenyl substituents the amine group is very weakly basic.

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CHAPTER FOUR

SPECTROPHOTOMETRY

4.1 Theory

4.1.1 Introduction.

Spectrophotometry, the measurement of the intensity of light absorption in a particular spectral region, is widely applicable, and is especially useful when substances in liquid medium has a strong characteristic absorption in a conveniently accessible region of the spectrum.[1]

The formation of a metal complex is often accompanied by a change in the light absorption of the complex relative to that of the individual metal ion or ligand.[2] A change in the visible spectrum is often attributed to the formation of inner-sphere complexes, whereas changes in the charge-transfer UV. region is associated with outer-sphere interaction or ion-pair formation. Changes in the ultraviolet region however may also reflect the presence of species formed in less close interactions and so the method is in principle, capable of distinguishing between outer- and inner-sphere ion-pairs (only if both regions can be theoretically be observed).[2]

In purely practical terms, the most important use of electronic spectroscopy in the UV and visible regions is in identification and quantitative measurement of certain transition metal complexes, which usually have bands of characteristic frequency and intensity in this region.[3]

Copper(II) is the most stable form of copper in aqueous solutions in its d^9 configuration which gives rise to Jahn-Teller distortions both in simple compounds and in complexes.[4] A wide range of stereochemistries is exhibited by Cu(II) compounds, with hexa-, penta-, and tetra-coordinate species. In aqueous solution, hexa-coordination is always attained by coordination of water molecules.[4]

Because of the Jahn-Teller effect, hexacoordinate Cu(II) species are always appreciably distorted with respect to the regular octahedral structure. The tetragonally distorted octahedral complexes of Cu(II) are blue or green, the blue aquo-ion being formed when Cu(II) salts are dissolved in an excess of water. It is tetragonally distorted, with two water molecules further away from copper than the other four, which are in a square plane around the metal.[4] A broad absorption in the 500-900 mμ region of the spectrum appears which is due to overlapping d-d bands. The intense bands in the UV region are due to ligand-metal charge transfer (LMCT) or intra-ligand transitions.[5]

4.1.2 Colour and Molecular Structure.[6]

The visible region of the electromagnetic spectrum is defined in terms of the wavelength range to which the human eye responds. The usual range of wavelengths quoted is from 380nm at the violet/blue end to 780nm at the long wavelength red end of the spectrum.[6] Beyond the short wavelength (violet/blue) end of the visible region there are the UV and X-ray regions, and beyond the long wavelength (red) there are IR, microwave and radiowave regions.

The main requirement is knowledge of the range of wavelengths over which the analyte solution absorbs. If the solution is coloured then we immediately know that it absorbs over the visible range and hence an instrument operating over the visible region is probably sufficient for structural analysis.[6]

4.1.3 Absorption Spectra with Molecular Structure.

When molecules interact with radiant energy in the visible and ultra-violet (UV) regions, the absorption of energy results in the excitation of an outer electron in the molecule. Rotational and vibrational modes are found combined with electronic transitions. Broadly, the spectrum is a function of the whole structure of the substance rather than of specific bonds.[7]

In reality atoms absorb and emit radiation and have their energy levels perturbed by external electric and magnetic fields.[8] As no unique electronic spectrum is found, this is a poor region for product identification by the fingerprint method.[9] On the other hand, electronic absorptions are often very intense. Molar absorptivity values frequently exceed 10 000 in the UV region, whereas in the IR region they rarely exceed 1000. Thus dilute solutions containing complexes are more easily measured in visible- UV spectrophotometry.[7]

4.1.4 The Absorption Spectra of Transition Metal Complexes.[10]

UV/Visible spectroscopy is useful in analysis of transition metal complexes, which absorb characteristically in the visible region of the electromagnetic spectrum (ems).[11] Over this range of wavelengths, absorbance is measured in an attempt to find the wavelength of corresponding to the d-d electronic transitions.[12]

The energy of electronic transition ($E = hc/\lambda_{max}$) whereby h:..., c:..., λ:... depends on the chemical environment of the metal ion and the position of λ_{max} will shift either towards longer or shorter wavelengths depending on the ligand attached to the metal ion. A shift towards longer wavelengths is called bathochromic or red shift and a shift towards shorter wavelengths is called hypsochromic or blue shift.[13]

Colour solutions are chromophoric[11] and are a result of absorption properties therefore the transmittance of light of a colour are complementary to the colour of the absorbed light of a certain wavelength.[14] Taken over a wider range of wavelengths, the spectra of transition metal complexes exhibit metal –ligand (or vice versa) charge transfer (LMCT) bands at about 200-700nm.[1]

The charge transfer bands are generally of very high intensities, which cause an electron to move from one descrete transition to another. The d-d transitions are characteristic of the transition metal. Transitions that are forbidden are between those energy levels that are of the same symmetry (g--->g, or u--- >u) or of the same orbital angular momentum quantum number (l). Since this is the case in the octehedral ligand field, the d-d transitions are not strictly allowed. They are said to be Laporté-forbidden.

The transitions are forbidden because electronic transitions have to involve a change in parity. Thus transition can only be allowed if the symmetry of the molecule is removed. If this is not the case, the transitions will be very weak, with an oscillator strength (f) of ~ 10^{-4}. The f is the effective number of electrons that are put into oscillation by the radiation field.

In the tetrahedral ligand field environment there is no centre of symmetry and therefore the orbitals may acquire some p-orbital character, so that the forbidden d-d transitions will be partially allowed. Distortion from perfect octahedral symmetry increases the transition probability and hence the intensity of the transition. This lowering of the symmetry of the molecule could be due to vibration, steric strain or an inhomogenous ligand field.[1]

When a metal ion contains only one d-electron, it is easier to predict the spectra to be expected from each ligand field. This is because the electronic distribution in the orbital will be dictated only by the ligand strength. However, when the metal ion contains more than one electron in the d-orbitals, inter-electronic repulsions have to be taken into account and this results in different electron spin-angular momenta combinations.[1]

In such cases, an initial comprehension of the different possible metal electronic orbital filling possibilities in the ground and excited states is important. How these will be affected by the symmetry of the resultant complex should follow from their initial distribution in the d-orbitals. Orgel diagrams are useful in showing the nature and energies of electronic transitions induced by ligand fields of different strengths and symmetries.[11]

When the electronic nature of the central ion is known, electronic spectra with characteristic absorption peaks and intensities can be used to suggest the nature of the ligands surrounding the metal ion.[15] Even though the structural information obtained from the electronic absorption spectroscopy is not as complete as that which would be obtained by diffraction methods, it does help in the structural elucidation of metal complexes especially for those in solution.

Some transition metals, including Cu(II) have unsymmetrically filled d-orbitals. Due to the Jahn-Teller effect the result of this is the tendency of the distortion of the octahedral geometry resulting in an uneven ligand field symmetry. These so called Jahn-Teller distortions of the regular octahedral coordination geometry can occur by either the shortening or lengthening of the axial ligand bond lengths.[15]

This distortion of the ideal octahedral symmetry causes a relaxation in the electronic selection rules resulting in more transitions. The energy of the absorption, the shapes of the absorption bands and their intensities can be very informative, especially when compared to the spectra of other related compounds.

A metal ion in a biological system encounters various kinds of donor atoms that have different ligand field strengths. These can include for instance the amino nitrogen, the peptide nitrogen, the imidazole nitrogen, the carboxylate oxygen, the peptide oxygen, the water oxygen and the hydroxide oxygen.[15] The ligand field strength of each of these different types of donor atom is further influenced by other factors such as the structure of donor molecule, the surrounding biological system and the metal that is being complexed at any particular time.

The energy contributions made by each donor atom to the total energy of the electronic transition of the metal complex can be calculated by a method proposed by Billo.[16] This is calculated by the formula:

$E = h\,\nu_{calc}$, whereby $\nu_{calc} = \sum \nu_i$ ------1

where ν_i is the contribution of atom i.

Based on the experimental work on about 34 copper complexes, Billo[16] proposed the following energy contributions for the different types of oxygen and nitrogen donor atoms:

$\nu(NH_3) = (4.53 \pm 0.07)\ X10^3/cm.$

$\nu(H_2O \text{ or } OH^-) = (3.01 \pm 0.03)\ X10^3/cm.$

$\nu(\text{carboxylate}) = (3.42 \pm 0.10)\ X10^3/cm.$

$\nu(\text{peptide } N_2) = (4.85 \pm 0.04)\ X10^3/cm.$

It is understood from this that the effects of the rest of the atoms of the ligand molecule are negligible.

4.1.5 The Absorption Spectra of Copper Complexes.

The chromophoric nature of the Cu(II) ion is due to its low lying d-orbitals.[4] The characteristic d-d absorption band which is in the visible region changes its transition levels according to whatever ligand field is around it.[11] The absorption spectra of transition metal complexes have been shown to be pH dependent. There is normally a general hypsochromic shift of the d-d absorption maximum that accompanies an increase in pH. This phenomena could be explained as an increase in the basicity of the donor atoms or/ as well as a possible increase in the number of coordinated donor atoms.[12]

The spectra of complexes in solution appear as broad spectral bands due to the unresolved rotational and vibrational absorption peaks.[18] The single band observed in the case of the Cu(II) complexes is composed of three absorption bands, corresponding to the following electronic state transitions:[12]

$^2A_{1g} \leftarrow {}^2B_{1g}$

$^2B_{2g} \leftarrow {}^2B_{1g}$

$^2E_{1g} \leftarrow {}^2B_{1g}$

When surrounded by six water molecules, Cu(II) shows maximum absorption at 800nm.[12] This maximum is moved to shorter wavelengths of approximately 600nm by the substitution of four of these water molecules by ammonia.[12]

The shift can be explained as follows: the enhanced splitting of the degenerate d-orbitals by the stronger ligand field of the nitrogen atoms causes the blue shift. The colour of the tetraammine aqueous solution is blue. Due to the Jahn – Teller effect, the four metal amine bonds are in one plane and they are shorter than the axial metal ligand bonds, which lie along the z-axis. A fifth ammine group can be added at one of these positions but it will be loosely bonded. The sixth position is even more weakly coordinated and $[Cu\ (NH_3)]_6^{2+}$ only forms in liquid ammonia.[17]

The nature of electronic transitions that are brought about by a chelating ligand can be inferred from general observation of the effect of monodentate ligands on the ligand field parameter[15] as described in Table 5. [16] This shows a guideline that is normally used for the estimation of the effect of the ligand field imposed by nitrogen based chelating ligands.

Table 5: The absorption maxima of the Cu(II) ammine complexes[19] *of the general formula* $[Cu\ (NH_3)_n\ (H_2O)_{6-n}]^{2+}$

N	Absorption maximum (nm)
0	790
1	745
2	680
3	645
4	590

Copper peptide complexes that are similar in structures tends to have similar λ_{max} values which implies that the energy of their electronic transitions is not significantly different.[20] During the formation of these complexes the colour changes from blue to purple to red in order of the increased number of coordinated nitrogen atoms.

The general approach to the study of metal complex spectra is by the "rule of average environment".[18] According to this rule the ligand field in a mixed ligand complex is assumed to be the average of the ligand fields of homogenous complexes of the constituent ligands. The reality is, biological metal complexes are hardly ever homogenous and hence this device has to be used to make coordination predictions in structural elucidation studies.

In solution, water molecules normally occupy the axial positions of copper complexes. The coordination of other ligands at these sites has an additional influence on the energy of the d-d electronic transitions. The presence of the fifth atom in the coordination sphere of the metal ion results in what is called the penta-ammine effect. A bathochromic shift is brought about by two axially coordinated sites. The presence of these groups in the coordination sphere results in the partial loss of the degeneracy of the orbital and the overall crystal field splitting energy of the metal complex is reduced, hence the "red" shift.[18]

4.1.6 Spectral Data Analysis.

The Beer-Lambert law[19] states that the absorbance (A) of light by a sample is directly proportional to the pathlength and the concentration of the absorbing species represented as:

$A = \varepsilon c l$ ----2 ε: Abs coefficient

This is a straightforward relationship if the sample contains only one absorbing species. In the case of complexometric titration solutions, there may be many absorbing species in the solution and so the A of a sample of the reaction mixture will be dependent on the concentrations of all the component species and their characteristic absorption coefficients Equation 2 will then become:

$A = l(\varepsilon_1 c_1 + \varepsilon_2 c_2 + \varepsilon_3 c_3 + \ldots \varepsilon_n c_n)$ ----3

$= l \Sigma \varepsilon_i c_i$

At different pH values the concentrations of different species will vary and so will A. This can be represented as:

$A_{HI} = l(\varepsilon_1 c_{1HI} + \varepsilon_2 c_{2HI} + \varepsilon_3 c_{3HI} + \ldots \varepsilon_n c_{nHI})$ ---- 4

$= l \Sigma \varepsilon_i c_{iHI}$

.
.
.

$A_{Hx} = l(\varepsilon_1 c_{1Hx} + \varepsilon_2 c_{2Hx} + \varepsilon_3 c_{3Hx} + \ldots \varepsilon_n c_{nHx})$ -------5

$= l \Sigma \varepsilon_i c_{iHx}$

where A_{Hl} is the total measured A at the first pH value and the $\varepsilon_1 c_{1Hl}$ is the A of species i at the first value of pH. The same applies to the subsequent values of pH up to the x^{th} (equation 7), for all the species up to the n^{th} species. If the concentration of the absorbing species at each pH value is known (c_{iHx}) then the set of simultaneous equations can be solved for ε of each species. Further, if at each value of pH, the A is measured over a range of wavelengths the total A of the sample at each wavelength will be:

$$A_{\lambda l} = l\,(\varepsilon_{1\lambda l}c_1 + \varepsilon_{2\lambda l}c_2 + \varepsilon_{3\lambda l}c_3 + \ldots \varepsilon_{n\lambda l}c_n) \quad \text{----6}$$

$$= l\Sigma\varepsilon_{i\lambda l}c_i$$

.
.
.

$$A_{\lambda x} = l\,(\varepsilon_{1\lambda x}c_1 + \varepsilon_{2\lambda x}c_2 + \varepsilon_{3\lambda x}c_3 \ldots \varepsilon_{n\lambda x}c_n) \quad \text{----7}$$

$$= l\Sigma\varepsilon_{i\lambda x}c_i$$

where $A_{\lambda l}$ in equation 6 is the total A at a specific wavelength and $\varepsilon_{i\lambda l}c_i$ is the A of species i at this wavelength. The range of equations for total A can be solved iteratively for the value of ε for each of the component species of the chemical system. The calculations of the solutions for ε can be done using a specially designed computer programme that requires an input file with all the information relevant to the chemical system being studied.[19]

The information includes amongst other parameters the concentration values for each of the chemical species present in the reaction solution. These concentration values are calculated on the basis of the stability constants found by glass electrode potentiometry.[19] The values of ε are then plotted against wavelength to give a deconvoluted spectrum showing the absorption band of each chemical species.

If the stability constants and hence the concentrations are not correct, the deconvoluted spectrum will be disjointed and this is indicative of a wrong chemical model. Having electronic absorption spectral information for each of the complex species thus affords a way of inferring the nature of the ligand field environment of the central metal ion.

The spectrophotometric method assists in the confirmation of a chemical model proposed by some other means such as potentiometry, and simultaneously in finding the coordination geometry of the component species of each models.[20]

4.2 Experimental Spectroscopy

4.2.1 Instrumentation and Chemicals

HP 8452A Diode-Array Spectrophotometer

15mm Cell/Cuvette

pH meter

Combined glass electrode

Acid	0.1M HCl ampoule
Base	0.1.M NaOH ampoule
Background Electrolyte	0.15M NaCl

4.2.2 Preparation of Solutions

The solutions for the UV/Visible spectroscopy were prepared as in potentiometry. The solutions for the nuclear magnetic resonance (NMR) studies were prepared in D_2O instead of distilled water. Solutions prepared were acid, base, metal ion both β-CD and 50%MeOH/H_2O were used in the solutions of the ligand. The chemical shifts in NMR studies were referenced using tertiary Butyl alcohol (t-BuOH).

4.3 Experiments

4.3.1 UV/Visible Spectrophotometry

4.3.1.1 CuGly conditions

In order to demonstrate that the experimental and data analysis procedures work the well-known Cu\Glycine system was studied. The concentration of the ligand was maintained at 0.01M at 1:2 fixed metal to ligand (M:L) ratio. The experiments were carried out in base increments and a distinctive colour change was noted as the experiments progressed. This was because the speciation of the glycine solution changes with pH and the different complex species have different absorption spectra. Water was used as a reference.

4.3.1.2 DCL conditions.

The concentration of the ligand was maintained at 8 $x10^{-4}$M as in potentiometric titration where the working pH range was obtained. The experiments were carried out in 50% MeOH/H_2O from high to low pH values, as the ligand forces the use of acid instead of base. Methanol was used as reference in these titrations.

4.3.1.3 CuDCL conditions.

The concentration of the ligand in these experiments was also maintained at 8 $x10^{-4}$M as in potentiometric titration where the working pH range was obtained. A 1:2 mole ratio of the M:L was maintained. Methanol was used as reference for these titrations with the acid increments.

4.3.2 Data Analysis for CuGly data.

Only the CuGly data could be analysed because of its chemical shifts. These data were analysed by the UV-spectra programme for the analysis of the molar absorptivity (ε), being the characteristic of the molecular electronic spectra. The input file to this programme specified include:

- The number of pH points investigated.
- The number of chemical species proposed including the metal ion.
- The number of wavelengths at which absorbance readings were taken at each pH.

These were then followed by:

The concentrations of the individual chemical species at each pH. Calculations of these concentrations were performed by the use of the programme SPEC (being a task to calculate the speciation of the species as a function of pH) found in ESTA.

Lastly as input were :

The recorded absorbance values of the titration solution at each pH over the whole range of wavelengths.

4.3.3 Other Studies.

Because of the failure of UV/Visible spectroscopy to give meaningful results on copper complexation of DCL, NMR and blood plasma modelling were done.

4.3.3.1 NMR Spectroscopy

NMR spectroscopy has been investigated for this particular ligand diclofenac. The purpose of the study was to see where exactly the metal ion Cu(II) would complex or bind to the ligand, be it to the amine group (in-between the phenyl groups) or the carboxylic group. Because of the low solubility of DCL the aim of this additional study was not fulfilled either as the technique requires high concentrations.

4.3.3.2 Blood Plasma Modelling

Metal ions play an important role in biological systems. Metals like Ca^{2+}, Mg^{2+}, Mn^{2+}, Fe^{2+}, Co^{2+}, Cu^{2+} and Zn^{2+} are considered to be essential for health in humans.[24] There are also those metals that are considered to be toxic to the human system. These are Pb^{2+}, Hg^{2+} and Cd^{2+}, which tend to compete with the essential metal ions for binding sites on the ligands of blood plasma. Therefore, it is not presently possible to set up a single simulation of blood plasma because of large number of compositions found in the plasma,[25] as the model contains all the species present in blood plasma. [27]

So the concept of plasma mobilising index (p.m.i) is used to study the selectivity of the ligand towards Cu(II), the p.m.i being defined as the ratio of the total concentration of low molecular weight (l.m.w) metal complex species in the presence of the drug to that of the normal plasma.[26] In addition, this p.m.i is a measure of the ability of the administered ligand to move metal ion to the l.m.w fraction.

The model also functions by assessing a particular system under *in vitro* conditions at manageable concentrations. The effects are then diluted to *in vivo* conditions by using appropriate mass balance equations.[26] So to understand the effect of the ligand on the equilibria, formation constants for the ligand together with the species determined *in vitro* are entered in the ECCLES (Evaluation of Constituent Concentration in Large Equilibrium Systems)[27] computer program

4.4 Results.

Table 6 below shows the results of CuGly in UV/Visible.

Table 6: *λ_{max} (nm) corresponding to ε ($mol^{-1}dm^3cm^{-1}$) for various Cu(II) species formed in solution with Glycine with data of stability constants from preceding chapter.*

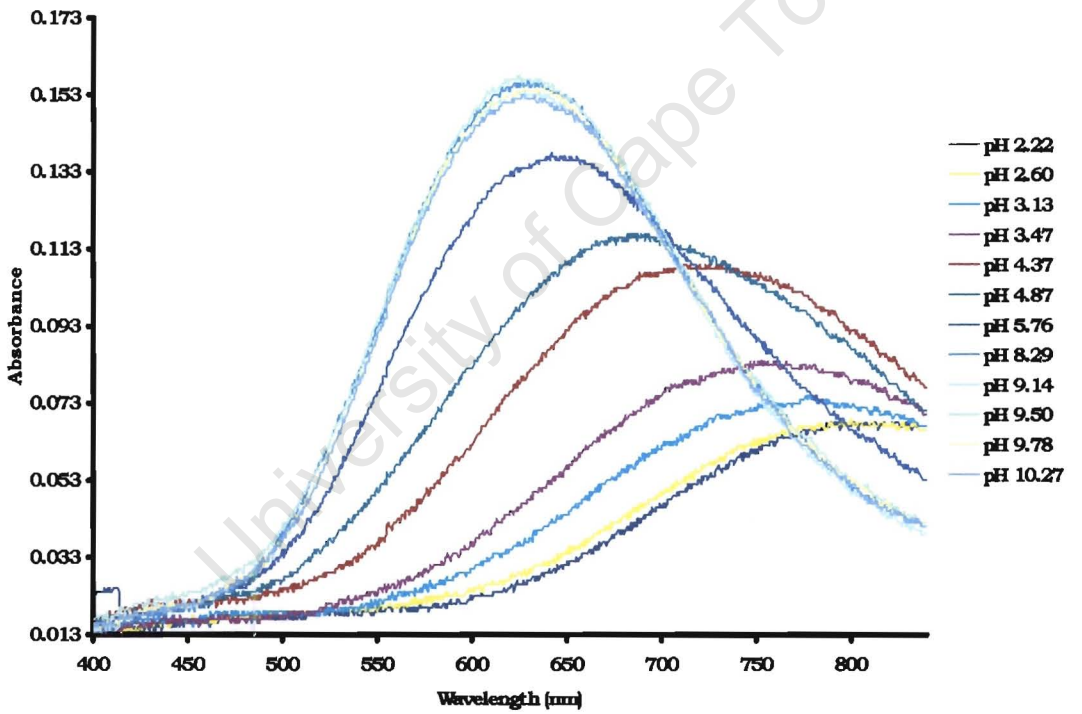
		λ_{max} (nm)	ε ($mol^{-1}dm^3cm^{-1}$)	β_{pqr}
M L	1 1 0	690	32	8.17
M L_2	1 2 0	650	42	14.95
M	1 0 0	780	16	-

4.5 Discussion

4.5.1 CuGly system

Because copper forms coloured complexes with glycine the solution structure of the complexes could be studied by UV/Visible spectrophotometry. Figure 12 below shows the UV/Vis. spectra of CuGly system as a function of pH.

Figure 12 UV/Visible Cu (0.001M)-Glycine (0.01M)Spectra

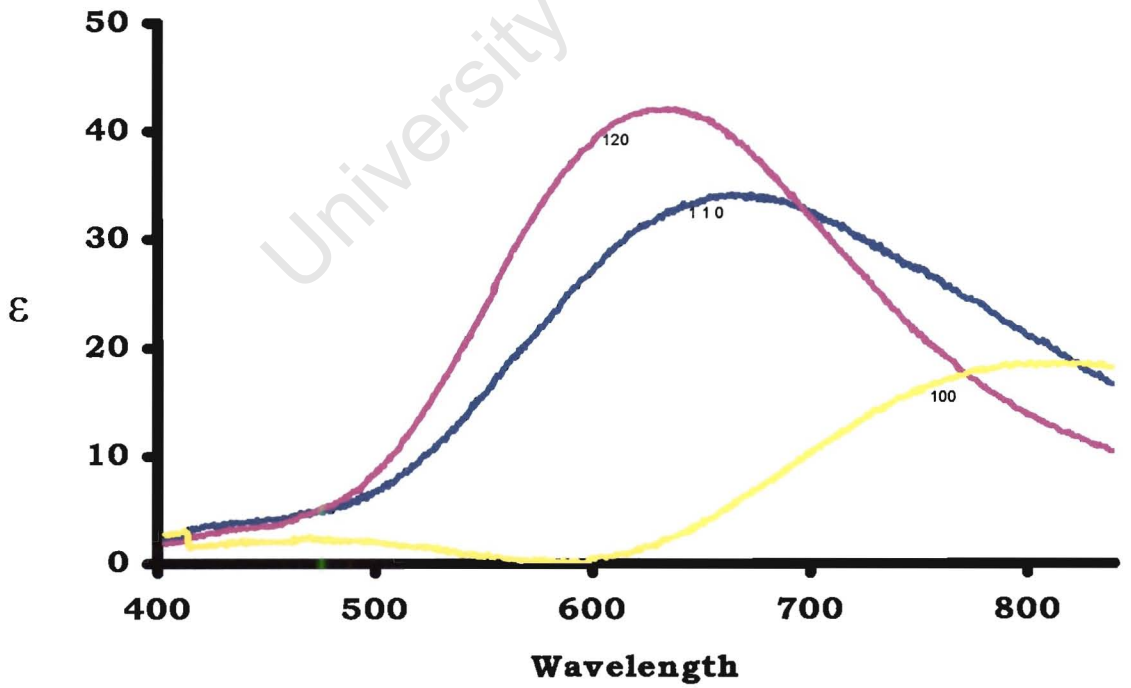


As can be seen, at each pH, a single peak is observed. As the pH is increased there is a bathochromic shift in λ_{max} and the intensity of the peak increases. This shift indicates increased ligand field splitting of the copper resulting from a change in the coordinated donor atoms.

In order to determine the structures of the individual species we need their spectra. Using deconvolution techniques as explained in section 4.3.2, the spectra in Figure 13 are obtained. The spectrum of the hexaaqua copper complex is as expected.

Cu(II) exists as the 1 1 0 species between pH 3-8 with a λ_{max} of 690nm. ε of this species is 32 $mol^{-1}dm^3cm^{-1}$ which is typical of Cu(II) in an octahedral environment. The ligand at λ_{max} of 650nm is doubly coordinated to the metal ion and ε in this complex is 42 $mol^{-1}dm^3cm^{-1}$.

Figure 13. Spectrum of Cu/Gly species.



4.5.2 DCL system

The problem of precipitation below pH 5 when βCD medium was used forces the use of 50% MeOH/H_2O medium. Firstly, before acid increments were added to the solution, the ligand was scanned for absorbance in the wavelength range selected (see Figure 14 below).

Figure14. UV/Visible Diclofenac (0.008M) Spectra.

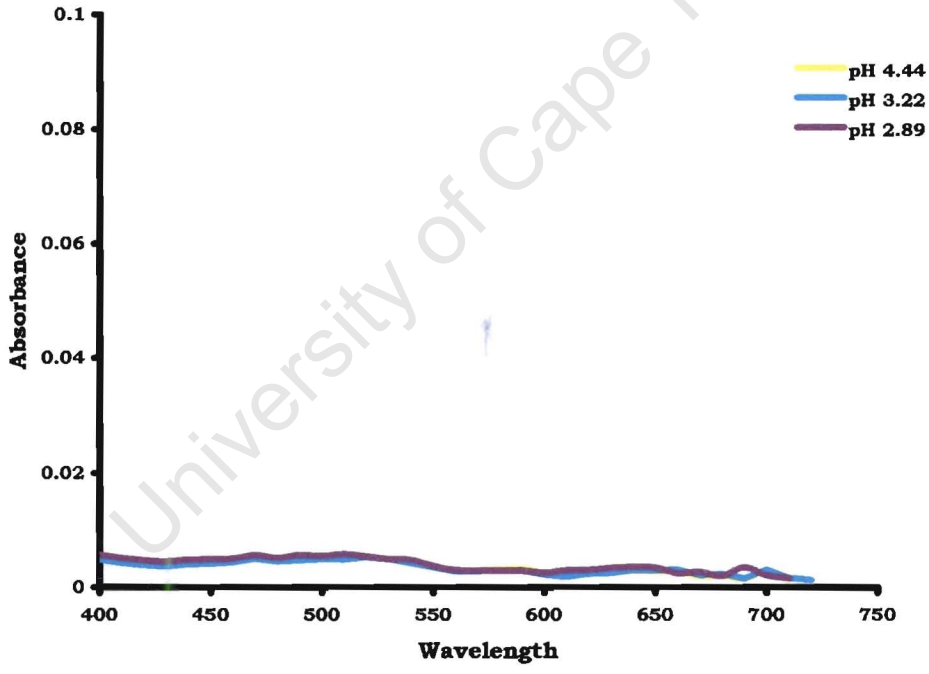


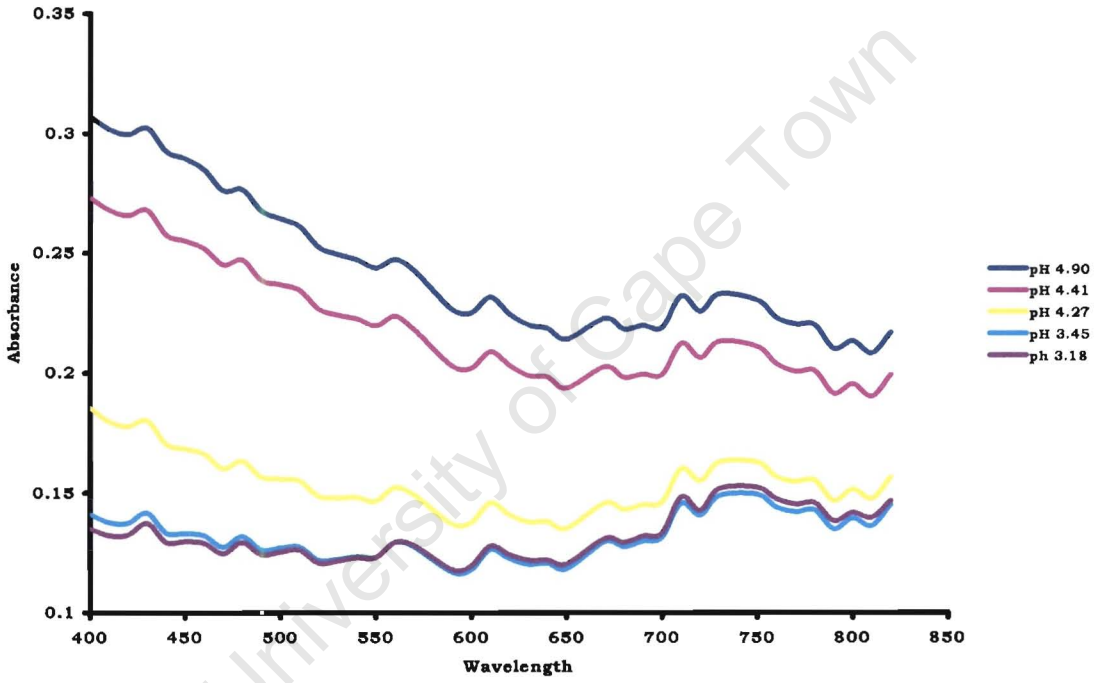
Figure 14 above is the spectrum of the ligand DCL in 50%MeOH/H_2O. At this concentration there was no visible absorption.

4.5.3 CuDCL system

For UV/Visible spectrophotometry, it was impossible to get any peaks in the region of the metal ion due to the weak nature of the complex formed.

The spectrum of a ligand together with a metal ion is shown in Figure 15 below. The purpose of this study was to check the structure of the complex formed when a copper metal ion was added to the ligand.

Figure 15. UV/Visible Cu(0.0002M-Diclofenac (0.0008M)Spectra.

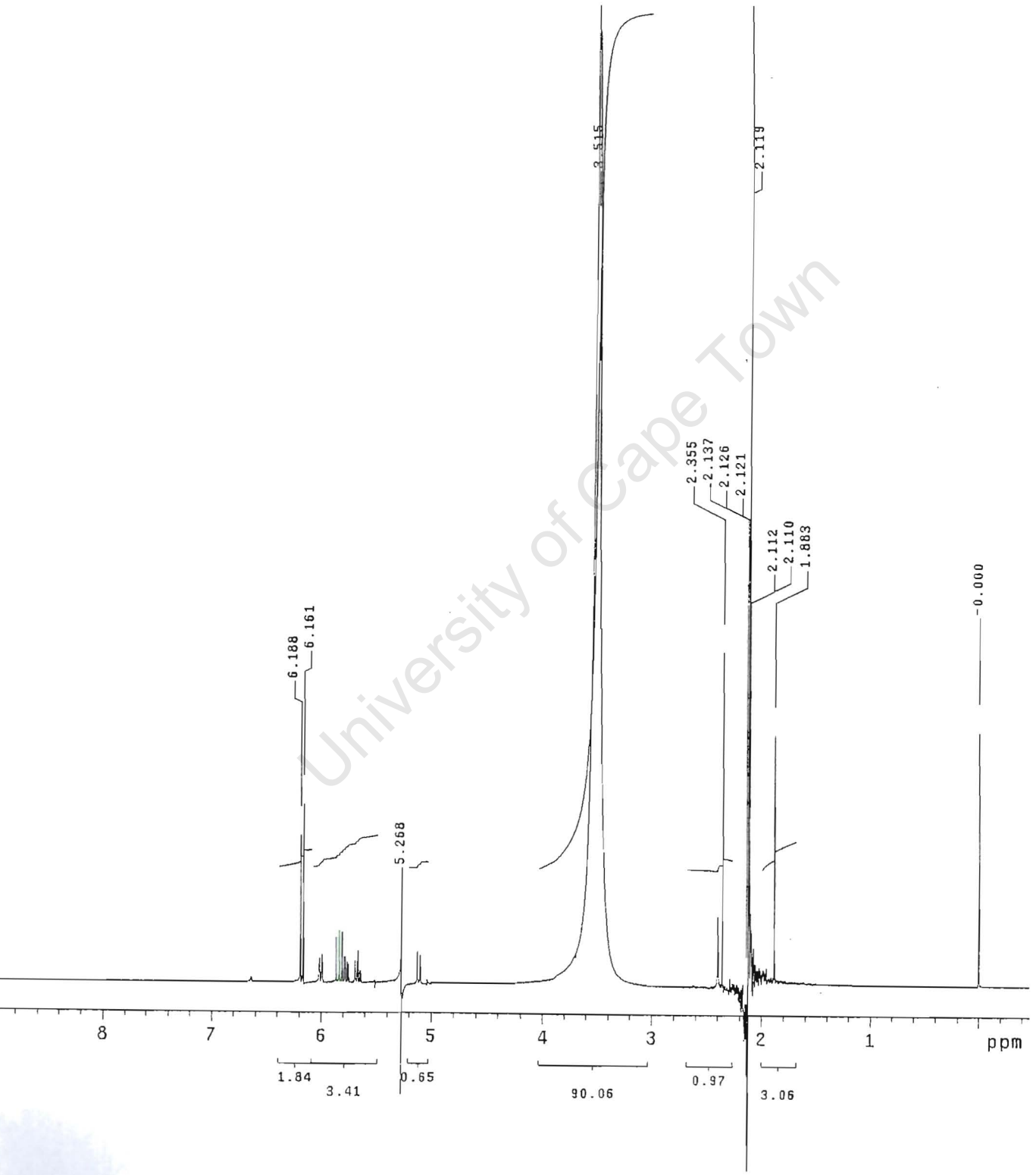


4.5.4 NMR spectroscopy

4.5.4.1 NMR study in 50%MeOH/H2O

The spectrum of a 6 x10^{-2}M solution of DCL in D_2O at pH 8.3 is shown in Figure 16 below. However, once again as the pH was lowered the ligand precipitated. Also after addition of the metal ion a white precipitate ($Cu(OH)_2$) was formed. For this reason it was not possible to obtain NMR spectra of sufficient quality in 50% MeOH/D_2O solution.

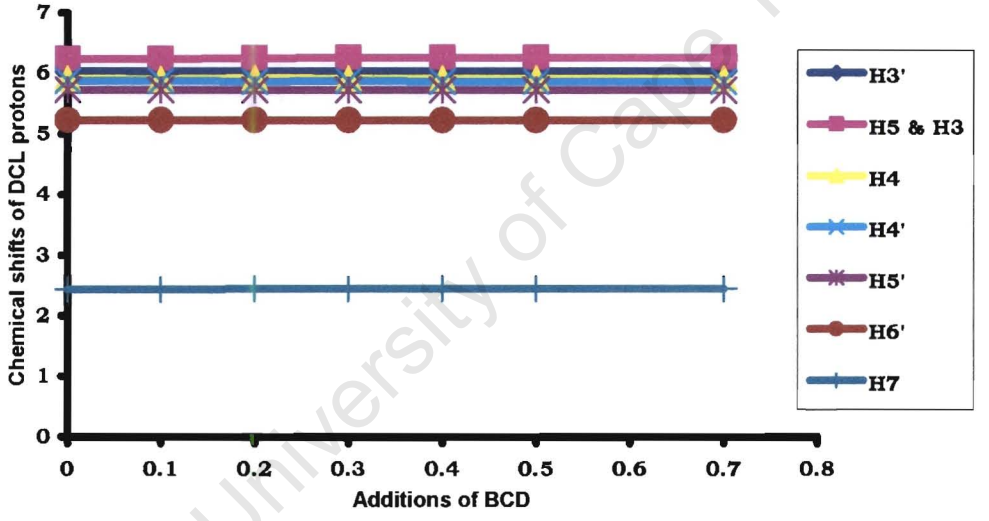
Figure 16. NMR Spectra for DCL in 50%MeOH/ H_20 at conc. of 6 x10^{-2}M.



4.5.4.2 The NMR study in β-CD.

Solubility of DCL in 50% MeOH/D_2O was insufficient to obtain reasonable NMR spectra. For this reason β-CD was used in an attempt to increase the solubility. Figure 17 below, shows the proton chemical shifts of DCL as a function of β-CD concentration.

Figure 17. NMR data analysis of DCL protons in β-CD .



The results obtained when β-CD was used were then compared with previously published results. J.A. Arancibia[23], managed to observe a downfield shift of approximately 0.05ppm in the resonances for protons in the phenyl ring holding an acetate group, in his studies performed in DMSO. This then provides him with evidence that this phenyl ring is the only molecular moiety involved in the complex formation between DCL and β-CD.[23] But in our experimental analysis performed in D_2O there was no change in chemical shifts.

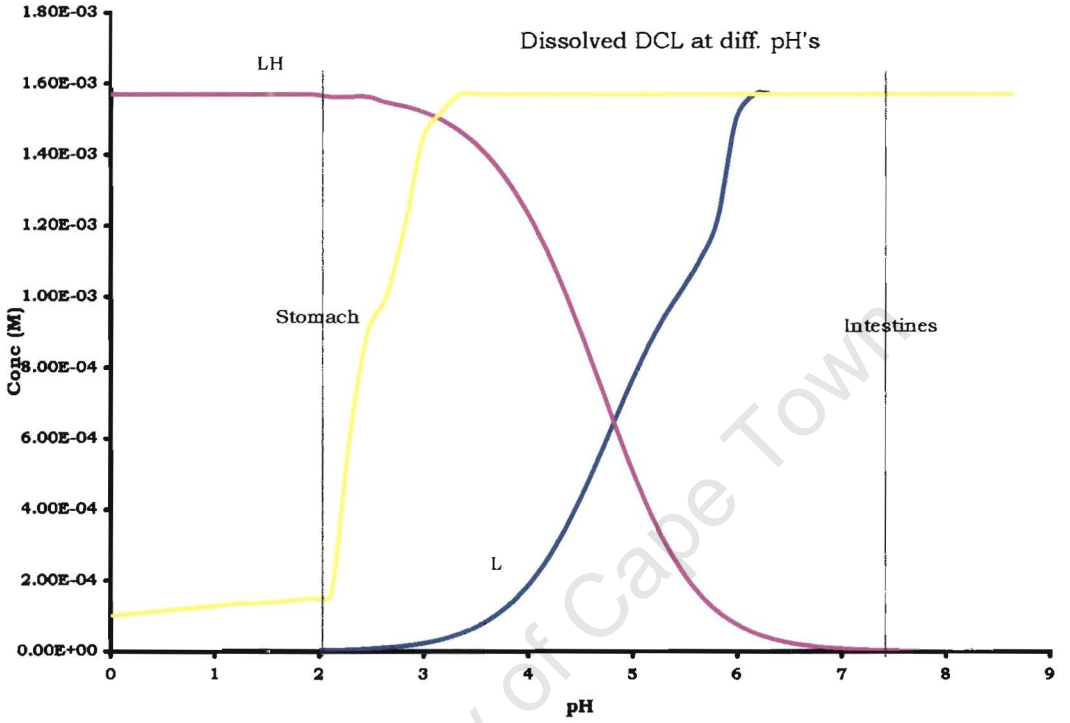
4.5.5 Computer Speciation Modelling

4.5.5.1 Intestinal Absorption

Diclofenac is administered orally in a tablet form with a dosage of 50mg or 75mg. The volume of a human stomach as outlined by G. B. Johnson[31] ranges from 4 L ~ 4000ml (full stomach) to 0.08 L ~ 80ml (empty stomach). Then if one assumes an average stomach volume is 150ml then it corresponds to a concentration range of 1.05 $x10^{-3}$M to 1.57$x10^{-3}$M. In the alimentary canal the DCL encounters a variety of pH conditions where in the stomach it is subjected to a pH of 2 while in the intestines the pH is ~7.8.

At a total DCL concentration of 8 $x10^{-4}$M in 50% MeOH/H_2O medium, the titration data showed that protonated DCL precipitates below pH 3.1. Using a protonation constant of 4.82 for DCL a solubility product of 5.5 $x10^{-8}M^2$ for the protonated DCL can be calculated. Figure 18 shows the speciation of DCL as a function of pH at a total concentration of 1.57 $x10^{-3}$M. On the same axes the total dissolved DCL is plotted.

Figure 18: Ligand speciation of 1.57 x10^{-3}M DCL as a function of pH

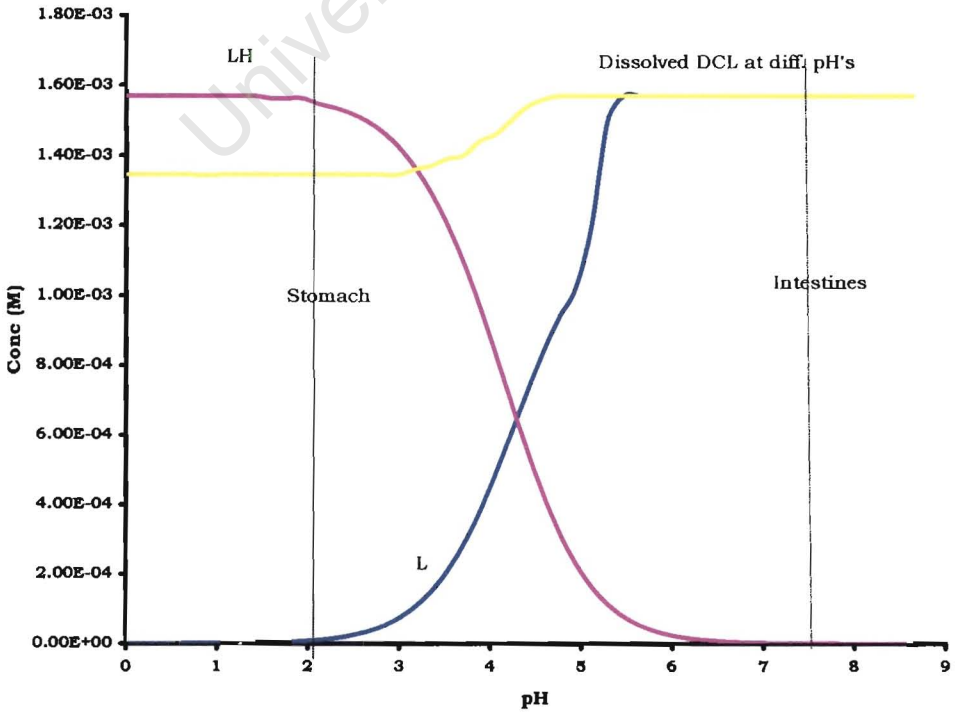


At pH 2, DCL is in the protonated form and only 9% of the ligand is dissolved. Above this pH there is a rapid increase in the amount of dissolved DCL. At the same time the DCL is deprotonated such that at pH 4.8 it exists as a 50:50 mixture of HDCL and DCL$^-$. At pH values of 6 to 8, typical of the intestines, DCL is exclusively in the deprotonated form and is fully soluble.

Pharmaceutical modification of drug molecules by inclusion in host compounds has been extensively developed to improve their solubility, chemical stability, absorption and bioavailability.[18]

A typical case is the microencapsulation of drug molecules in cyclodextrins (CD's) which has been observed to produce more stable drug preparations with improved bioavailability.[19] Our experimental data on DCL/β-CD system showed that at a DCL concentration of 8 $x10^{-4}M$ precipitation occurred at pH 4.5. Using this pH and using a protonation constant of 4.29 for encapsulated DCL, a solubility product of 6.9 $x10^{-8}M^2$ is calculated. Interestingly, there is very little difference between the solubility products of DCL in the two media. However there is a significant difference in the solubility. Figure 19 shows the speciation of DCL in β-CD medium as a function of pH.

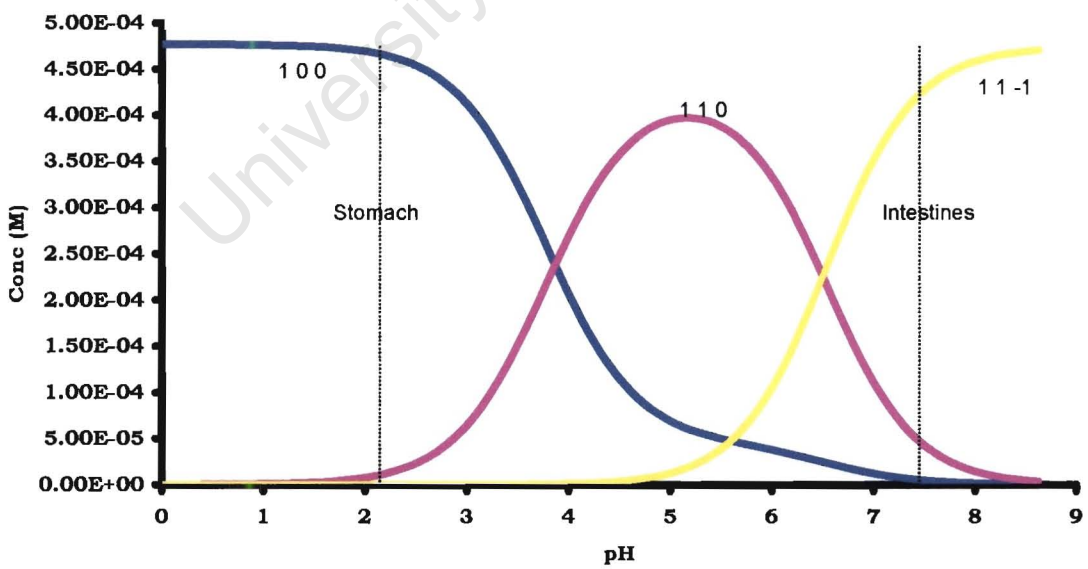
Figure 19 DCL (1.57 x10⁻³M) in β-CD speciation as a function of pH



The speciation plot confirms that the solubility of DCL has been improved to 1.35 x10^{-3}M at pH 2 compared to 1 x10^{-4}M around the same pH when methanol medium was employed. This is because of the difference in protonation constants.

The main aim of this study was to investigate the complexation of Cu(II) by DCL. Then, 50% MeOH/H_2O medium was used because of its better working pH range when compared to β-CD medium. Figure 20 shows a species distribution graph of CuDCL as a function of pH in 50% MeOH/H_2O.

Figure 20. Species distribution of CuDCL in MeOH as a function of pH.



Between pH 0 and 2, the free Cu^{2+} and the protonated DCL exist. The 1 1 0 species reaches a concentration of 4 x10^{-4}M at pH 5.5. Above pH 7 the 1 1 –1 species predominates.

These results show that even though Cu(II) is weakly coordinated to DCL, in the absence of competing ligands the complex would exist in the intestines. This may well affect the bioavailability of the DCL and the Cu(II).

4.5.5.2 The copper p.m.i

In the blood plasma there are other competing ligands found so the CuDCL complex was exposed to such kind of environment. In the calculation of the p.m.i of Cu^{2+}, the ligand concentration was varied over the range of 10^{-9} to 10^{-4} mol dm^{-3}. The stability constants were also entered into ECCLES program, the blood plasma model, to evaluate the bioavailability of the copper ions from complexes determined. The iterations were done at pH 7.4 and the log p.m.i for Cu^{2+} is constant at 0.00 throughout the concentration range. The log p.m.i is normally plotted against the log of the concentration of the ligand. A high p.m.i of any ligand indicates strong metal ion chelation at very low ligand concentrations. But it was impossible to plot the graph under these conditions.

The results shows that the ligand is devoid of any Cu(II) mobilising ability. The fact that the metal p.m.i is 0.00, this can be due to the weak complexes formed by Cu(II) with DCL. In conclusion, the DCL is unable to increase the l.m.w Cu(II) fraction.

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CHAPTER FIVE

CONCLUSION

5. Conclusion

The anti-inflammatory activity of metal complexes of NSAIDs has been known for many years. Previous studies have shown copper compounds to have anti-inflammatory activity.[1] The objectives in designing copper-based anti-inflammatory drugs has been to increase the concentration of the low molecular weight copper in plasma.[1] Although the mechanism of action by which copper exhibits anti-inflammatory activity is not yet understood, several possibilities exist.

Whatever its mode of action, the importance of copper at the site of inflammation has been sufficiently established and hence its manipulation by pharmaceuticals is essential. This therefore represents the justification for studying Cu(II) complexes, particularly with respect to their copper mobilising ability.[2]

Furthermore much pharmacological evidence suggests the use of copper complexes to be beneficial in the alleviation and treatment of RA. Thus Sorenson[1] demonstrated that the anti-inflammatory activity of various drugs was increased when these were administered as their copper complexes.

Diclofenac, the ligand of interest is used to treat inflammation associated with RA. It has been postulated that the activity of this drug would be increased by complexation with copper. The objective of this study was to investigate this claim and to elaborate any underlying chemical basis for it.

For the ligand to meet the requirements of copper in vivo mobilisation, it must possess several thermodynamically desirable characteristics. These vary according to the particular metal ion being considered. Unfortunately, enhanced stability is not the only criterion for successful mobilisation, but selectivity as well as other physical attributes of the complex plays an important role.

There are several different ways of achieving selective thermodynamic binding. Of these the most important for controlling the selection of copper are the types of liganding donor atoms, the preferential co-ordination geometry and the metal ion to ligand size fit.

Because of solubility limitations the system was studied in 50% MeOH/H_2O and β-cyclodextrin (β-CD) as a carrier molecule and then protonation constants of 4.82 and 4.29 were obtained for the two media respectively. It was not possible to investigate the copper complex of the ligand using the β-CD environment.

The only log stability constants obtained were 3.48 and –3.02 for the 1 1 0 and 1 1 -1 species respectively in 50% MeOH/H_2O. Cu(II) shows a remarkable ability to form bonds at pH ~7 to peptide and amide nitrogens in their ionised state.[3] However in this study, as the amine nitrogen has a very low basicity and so, at pH > 4.8, the copper precipitates out of solution.

Based on pKa values the solubility products of 5.5 $x10^{-8}M^2$ and 6.9 $x10^{-8}M^2$ were calculated for the protonated DCL. The improvement in solubility products is due to the difference in protonation constants for the two media used. Computer simulation of intestinal absorption indicates that the CuDCL complex would exist at pH > 5. In this way the bioavailability of both copper and DCL is improved. However blood plasma simulation studies indicated that the ligand is devoid of any Cu(II) mobilising ability. This can be due to the weak complexes formed by Cu(II) with DCL relative to other endogenous copper binding ligands.

Also, the study enriches the knowledge of diclofenac with some aspects of its complexing ability and although the reaction medium is very different from the biological environment. However the obtained results enable to suppose that the administration of this drug for long time could decrease the bioavailability of some important metal ions. In fact diclofenac reacts with divalent metal ions forming 1:2 (metal to ligand ratio) very slightly water-soluble complexes. Therefore, DCL is unable to increase the l.m.w Cu(II) fraction.

Improvements in the stability of the ligand may be the increase in its number of electron donor atoms or a slight change in the whole ligand has to be done. Also if the number of chelate rings on the ligand can be increased that would enhance the stability of the system.

Chapter Five References

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